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# The Role of Cytosolic Access in Streptococcus Pneumoniae Nasopharyngeal Colonization

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# The Role of Cytosolic Access in *Streptococcus Pneumoniae* Nasopharyngeal Colonization

## Abstract

Cytosolic detection of pathogen associated molecular patterns is a key event in host discrimination between commensal and pathogenic microbes. While cytosolic access is critical for the pathogenesis of intracellular bacteria, access to the cytosolic compartment by extracellular bacteria is less well understood. The leading extracellular pathogen *Streptococcus pneumoniae* (the pneumococcus) activates intracellular innate immune responses, but unlike intracellular bacterial pathogens, *S. pneumoniae* is killed and degraded upon uptake by phagocytic cells. The pneumococcus serially colonizes the human upper respiratory tract and is eventually cleared over a period of weeks by the host immune response. Previous studies have defined a critical role for the cytosolic pattern recognition receptor Nod2 in driving the macrophage recruitment that leads to clearance of pneumococci, although the mechanism by which pneumococcal cell wall components access the cytosol to activate Nod2 signaling remains unclear.

In these studies, we demonstrate that cytosolic access of pneumococcal components is dependent on bacterial degradation by the host muramidase lysozyme and on the activity of the bacterial pore-forming toxin pneumolysin. We propose a model in which *S. pneumoniae* is phagocytosed by macrophages whereupon it is killed and degraded by lysozyme, allowing bacterial cell wall components to escape into the host cell cytosol via the action of pneumolysin on the phagosome membrane. We further define a host mechanism that limits the amount of pneumococcal products that transit to the cytosol, although this defense is insufficient and the macrophage undergoes pro-inflammatory cell death.

This cell death is triggered by the formation of inflammasomes, multi-protein cytosolic complexes, that activate caspase-1 to drive secretion of the pro-inflammatory cytokine interleukin-1 beta (IL-1 $\beta$ ). We show that sensing by the type 1 IL-1 receptor (Il1r1 $^{-/-}$ ) is required for the host to effectively recruit macrophages to the nasopharynx and that Il1r1 $^{-/-}$  mice have delayed bacterial clearance. Furthermore, sensing of IL-1 cytokines contributes to inflammation in the nasopharynx, which may promote *S. pneumoniae* growth or transmission, but does not impact the adaptive immune response of the host. This suggests that a bacterial toxin and the subsequent intracellular innate immune sensing it causes may help the pneumococcus promote its extracellular lifestyle.

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THE ROLE OF CYTOSOLIC ACCESS IN *STREPTOCOCCUS*  
*PNEUMONIAE* NASOPHARYNGEAL COLONIZATION

Jamie K. Lemon

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## **ABSTRACT**

### THE ROLE OF CYTOSOLIC ACCESS IN *STREPTOCOCCUS* *PNEUMONIAE* NASOPHARYNGEAL COLONIZATION

Jamie K. Lemon

Jeffrey N. Weiser

Cytosolic detection of pathogen associated molecular patterns is a key event in host discrimination between commensal and pathogenic microbes. While cytosolic access is critical for the pathogenesis of intracellular bacteria, access to the cytosolic compartment by extracellular bacteria is less well understood. The leading extracellular pathogen *Streptococcus pneumoniae* (the pneumococcus) activates intracellular innate immune responses, but unlike intracellular bacterial pathogens, *S. pneumoniae* is killed and degraded upon uptake by phagocytic cells. The pneumococcus serially colonizes the human upper respiratory tract and is eventually cleared over a period of weeks by the host immune response. Previous studies have defined a critical role for the cytosolic pattern recognition receptor Nod2 in driving the macrophage recruitment that leads to clearance of pneumococci, although the mechanism by which pneumococcal cell wall components access the cytosol to activate Nod2 signaling remains unclear.

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## LIST OF ABBREVIATIONS

<b>adr</b>	Attenuator of drug resistance, a pneumococcal enzyme that modifies the cell wall to confer resistance to lysozyme
<b>ALRs</b>	AIM2-like receptors, a family of cytosolic pattern recognition receptors
<b>ASC</b>	Apoptosis-associated speck-like protein containing a CARD, an adaptor protein for the inflammasome complex
<b>BAF</b>	Bafilomycin A, a specific inhibitor of the vacuolar ATPase
<b>BMMs</b>	Bone marrow derived macrophages
<b>CARD</b>	Caspase activation and recruitment domain, a conserved domain that facilitates signaling through homotypic interactions
<b>CCL2</b>	C-C chemokine ligand 2, a monocyte chemoattractant protein
<b>CCL6</b>	C-C chemokine ligand 6, a monocyte chemoattractant protein expressed in rodents
<b>CCR2</b>	C-C chemokine receptor type 2, a host receptor that binds CCL2
<b>CDC</b>	Cholesterol dependent cytolysin, a family of pore-forming toxins
<b>CFU</b>	Colony forming units
<b>ChoP</b>	Phosphocholine, a moiety that anchors choline binding proteins to the pneumococcal cell surface
<b>CLRs</b>	C-type lectin receptors, a family of membrane-bound pattern recognition receptors
<b>CPS</b>	Capsular polysaccharide, a virulence factor of <i>Streptococcus pneumoniae</i> , that is covalently linked to the bacterial cell wall and defines the 91 known serotypes of pneumococci
<b>CRP</b>	C-reactive protein, a host factor that targets pneumococcal choline binding proteins and activates the alternative complement pathway

<b>CytD</b>	CytochalasinD, an inhibitor of actin polymerization
<b>GBS</b>	Group B <i>Streptococcus</i>
<b>GMT</b>	Geometric mean titer
<b>IgA1</b>	Immunoglobulin A1
<b>IL-1<math>\beta</math></b>	Interleukin-1 beta, a pro-inflammatory cytokine that is matured and secreted following inflammasome activation
<b>IL1r1</b>	Type 1 interleukin-1 receptor, a host receptor that binds interleukin-1 alpha and interleukin-1 beta
<b>IL-6</b>	Interleukin-6, a pro-inflammatory cytokine
<b>IL-17a</b>	Interleukin-17a, a pro-inflammatory cytokine that is important for immunity to <i>S. pneumoniae</i>
<b>IL-18</b>	Interleukin-18, a pro-inflammatory cytokine that is matured and secreted following inflammasome activation
<b>IRAK-4</b>	Interleukin-1 receptor-associated kinase 4, a kinase that is activated downstream of TLR signaling
<b>LDH</b>	Lactate dehydrogenase, a host cellular enzyme
<b>LLO</b>	Listeriolysin O, the cholesterol dependent cytolysin of <i>Listeria monocytogenes</i>
<b>LPS</b>	Lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria
<b>MAPKi</b>	SB203580, an inhibitor of p38 MAPK
<b>MARCO</b>	Macrophage receptor with collagenous structure, a host scavenger receptor
<b>MCP-1</b>	Monocyte chemoattractant protein 1, an alternate name for CCL2
<b>MDP</b>	Muramyl dipeptide, the minimal motif of the bacterial cell wall that can activate Nod2
<b>MYD88</b>	Myeloid differentiation primary response gene 88, an adaptor molecule for both Toll-like receptors and the interleukin-1 receptor

<b>NETS</b>	Neutrophil extracellular traps
<b>NF-<math>\kappa</math>B</b>	Nuclear factor kappa-light-chain-enhancer of activated B cells, a transcription factor that drives pro-inflammatory cytokine production
<b>NLRs</b>	Nod-like receptors, a family of cytosolic pattern recognition receptors
<b>Nod</b>	Nucleotide oligomerization-binding domain, a characteristic domain of Nod-like receptors
<b>PAMPs</b>	Pathogen associated molecular patterns, conserved microbial motifs that are detected by host receptors
<b>PCV7</b>	7-valent pneumococcal conjugate vaccine, effective against 7 serotypes
<b>PCV13</b>	13-valent pneumococcal conjugate vaccine, effective against 13 serotypes
<b>PgdA</b>	Peptidoglycan N-acetylglucosamine deacetylase A, a pneumococcal enzyme that modifies the cell wall to confer resistance to lysozyme
<b>PRRs</b>	Pattern recognition receptors, host receptors of the innate immune system that detect microbial pathogens
<b>PspA</b>	Pneumococcal surface protein A, a virulence factor of <i>S. pneumoniae</i>
<b>PYD</b>	Pyrin domain, a conserved domain that facilitates inflammasome assembly through homotypic interactions
<b>qRT-PCR</b>	Quantitative reverse transcription polymerase chain reaction
<b>RIP2</b>	Receptor-interacting protein 2, an adaptor protein for Nod1 and Nod2
<b>RLRs</b>	(RIG)-I-like receptors, a family of cytosolic pattern recognition receptors
<b>Spn</b>	<i>Streptococcus pneumoniae</i>
<b>TLRs</b>	Toll-like receptors, a family of membrane-bound pattern recognition receptors

<b>TLR2</b>	Toll-like receptor 2, a host receptor that detects pneumococcal lipoteichoic acids
<b>TLR4</b>	Toll-like receptor 4, a host receptor that detects LPS and pneumolysin
<b>URT</b>	Upper respiratory tract

# **CHAPTER I**

## **Introduction**

## Historical Background

*Streptococcus pneumoniae* (the pneumococcus) was independently isolated in 1880 by both George Sternberg in the United States (252) and Louis Pasteur in France (209). Historically, Pasteur receives credit for the discovery because he published three months before Sternberg, although Pasteur's experiments were actually performed later (11). Both men injected human saliva subcutaneously into rabbits – for Pasteur the saliva was from a child and for Sternberg the sample was his own saliva – and thereafter recovered a lancet-shaped diplococcal bacterium from the blood of the inoculated rabbits. This organism was first referred to as *Pneumococcus* in 1886 (82) for its association with pneumonia, and in 1920 it was renamed *Diplococcus pneumoniae* to reflect both its cellular morphology and clinical presentation (281). In 1974 it received its current designation of *Streptococcus pneumoniae* (64) because it grows in short chains in broth culture, a defining characteristic of the *Streptococcus* genus. While initially isolated from a state of carriage, within a decade of the pneumococcus' discovery it had been recovered from cases of pneumonia (96, 153), septicemia (258), meningitis (198), otitis media (289), and even arthritis (274).

Although often studied for the variety of human diseases it can cause, perhaps the most important contribution the pneumococcus has made to our understanding of biology has little to do with its clinical manifestations in humans. In 1928 Fred Griffith published the now famous 'Griffith's

experiment' that demonstrated the principle of bacterial transformation (95). Griffith observed that injecting mice with an unencapsulated pneumococcal strain caused no disease, while the mice rapidly succumbed to infection following injection of an encapsulated strain. In his experiment, Griffith injected mice with a mixture of live unencapsulated and heat-killed encapsulated pneumococci. The mice quickly died and only live encapsulated organisms could be recovered. As an added layer of detail, Griffith further showed that the capsule type acquired by the live pneumococcal strain after injection was always the capsule type of the heat-killed strain, even if that capsule was a different type from the parental live strain. Avery and colleagues built upon these observations and were able to reconstitute the transformation reaction *in vitro* (3, 61, 62, 246). In their landmark paper Avery, MacLeod, and McCarty provided definitive evidence that DNA was the material responsible for the phenotypic changes observed during bacterial transformation (14). This was the first demonstration that a nucleic acid could have biologic functions and these findings eventually gave rise to the field of molecular genetics.

In addition to aiding our understanding of fundamental biology, research involving the pneumococcus has also led to discoveries of great clinical importance. Shortly after the identification of the pneumococcus, Christian Gram published his technique for staining clinical tissue samples to aid in the identification of pathogenic organisms (93). Gram examined samples from the lungs of 20 patients who had died of pneumonia. In 19 of

the specimens he observed cocci that had not decolorized after aniline-gentian violet staining (272), making *S. pneumoniae* the first identified Gram-positive organism. Interestingly, in the one sample that had no Gram-positive bacteria, Gram observed a bacterium that was readily decolorized (Gram-negative), which is known today as *Klebsiella pneumoniae*. The significance of this finding was not initially fully appreciated, resulting in confusion about which organism was the true etiologic agent, though the roles of both *K. pneumoniae* and the pneumococcus as causes of pneumonia were eventually established (9). Nevertheless, Gram staining remains to this day a primary means of pathogen identification in the clinical microbiology laboratory.

The pneumococcus was also used in the first demonstration of antibiotic resistance. In the beginning of the 20<sup>th</sup> century, researchers observed that *S. pneumoniae* was sensitive to the quinine derivative ethylhydrocupreine, also known as optochin, while similar bacterial species were not (187). As part of this work, mice were infected with the pneumococcus and then experimentally treated with optochin. The investigators rapidly isolated pneumococci that had developed resistance to optochin (186). This experiment is additionally noteworthy as it is one of the first examples of using a specific compound to treat an infection (272).



## **Treatment and Vaccination**

Early treatment for pneumococcal disease was based on findings from studies conducted in rabbits at the end of the 19<sup>th</sup> century. In these experiments it was observed that serum from rabbits previously infected with pneumococci could protect against a secondary challenge with the same bacterial strain (141). Later, it was established that the pneumococcal capsular polysaccharide was the antigen responsible for this seroreactivity (103), making it the first non-protein antigen to be identified (272), antisera could be separated by strain type. The method of typing pneumococcal strains used well into the 20<sup>th</sup> century, known as the Quellung reaction (199), involves incubating pneumococci with the specific antisera. The reaction is monitored for bacterial agglutination and swelling of the capsule, which occurs only when the strain and antisera are of the same type. Once strains could be reliably serotyped, patients with pneumococcal disease were successfully treated through passive immunization with sera raised in horses (80).

The success of serotherapy and development of antibiotics may have actually delayed advances in the development of a vaccine for the pneumococcus (272). The first vaccines against *S. pneumoniae* demonstrated that inoculation of purified capsule alone could generate protective immunity (76, 79, 159, 248) and that multiple polysaccharide types inoculated at once were also effective (104, 134). These early vaccines were removed from the market due to low usage (12) and were only

revisited decades later as a result of the work of Robert Austrian.

Austrian developed a 14-valent capsule vaccine and demonstrated its efficacy in preventing pneumococcal pneumonia (10, 13). The 14-valent vaccine was introduced in 1977 and eventually expanded in 1983 to include 23 capsule types (230). Today this is still the broadest coverage of any pneumococcal vaccine. However, the capsule vaccine had limited success in protecting children due to lack of immunogenicity (172).

Following the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the US in 2000, rates of both invasive pneumococcal disease and pneumonia decreased dramatically (28, 94). This success was due in large part because the vaccine is efficacious in young children, the demographic most likely to contract pneumococcal disease (44). Similar declines in pneumococcal-attributed childhood illnesses were also observed internationally (70, 123), including in developing countries (43, 52, 92). This is noteworthy because the relative distribution of pneumococcal serotypes varies with geographic location (176) and PCV7 targeted the seven most common serotypes in the developed world (249). Serotype coverage expanded with introduction of the 13-valent conjugate vaccine (PCV13) in 2010 (201), however this still represents only a fraction of the known pneumococcal serotypes that cause disease. In the years following vaccine introduction, researchers observed that non-vaccine serotypes began to circulate more frequently in the population (1, 107), a phenomenon known as 'serotype replacement.' The degree to which serotype replacement

threatens to undermine the advances that vaccination has made against pneumococcal disease is a subject of debate (30, 155), though current next-generation pneumococcal vaccines are focused on providing broader serotype coverage (90, 158, 290), ideally at lower cost (183). This is critical, as majority of the deaths attributed to pneumococcal disease today occur in the developing world (152, 202, 283).

Despite the success of the polysaccharide and conjugate vaccines, there were still an estimated 33,500 cases of invasive pneumococcal disease and 3,500 deaths due to *S. pneumoniae* infection in the U.S. in 2013 (44). The current treatment for pneumococcal infection is antibiotic therapy and like many other pyogenic bacteria, the development of antibiotic resistance remains a pertinent issue. The first patient to show clinical improvement from penicillin treatment was suffering from pneumococcal conjunctivitis (272) and the use of this antibiotic, as well as other  $\beta$ -lactams, remains common today. However, within a couple decades of widespread usage, penicillin-resistant pneumococci had been isolated clinically (100, 101). Rates of antibiotic resistance, specifically to penicillin, increased to over 25% in the U.S. (108), although evidence suggests that introduction of the pneumococcal conjugate vaccine has reduced levels of antibiotic resistance (53, 136).

The years following conjugate-vaccine introduction also saw decreased pneumococcal disease in the elderly (94), a population that had not received either PCV7 or PCV13. This 'herd immunity' - protection of an unvaccinated

portion of the population - is attributed to the ability of the conjugate-vaccine to reduce the carrier state in children (54, 180). Animal models have demonstrated that this decrease in carriage is due to vaccine-induced antibody blocking the acquisition of the pneumococcus in the upper respiratory tract (232). The ability of the conjugate-vaccine to confer herd immunity underscores a critical aspect of the pneumococcus' lifestyle - colonization and carriage in the hosts' nasopharynx.

### **Pneumococcal Nasopharyngeal Colonization**

*Streptococcus pneumoniae* serially colonizes the nasopharynx of healthy humans. Colonization results in a suppurative rhinitis that is characterized by increased mucus secretion. Recent animal studies have demonstrated that these secretions are important for pneumococcal growth (247) and are also believed to contribute to transmission (66, 228, 244). Humans are the only natural host for pneumococci, and epidemiologic studies show that transmission increases in high-density populations, such as daycare centers, nursing homes, prisons, or within households (110, 177, 193, 200, 224). Until recently there were no tractable animal models of pneumococcal transmission. The current model of pneumococcal transmission relies on viral co-infection with Influenza A (66) and it appears that the inflammation induced by viral infection is critical for transmission to occur (244). Although this model has advanced our understanding of

transmission, this aspect of pneumococcal pathogenesis remains vastly understudied.

Colonization events can occur as early as the first day of life, with the highest rates of carriage in children under the age of 2 (29). In this population, the rate of colonization is estimated at 20-50% (29), though some studies have reported carriage rates as high as 90% (71). Rates of colonization decrease with increasing age (156) and in adults carriage is estimated to be below 20% (29). Individuals can be colonized with multiple distinct pneumococcal strains at the same time (8, 37).

Following the establishment of colonization, pneumococci are subsequently cleared from the upper respiratory tract by the hosts' immune system. Clearance generally occurs over a period of a few weeks to a month (173). However, colonization with the same serotype for a duration lasting years has been reported (273). Nevertheless, this immune response is unable to confer protection to the host and individuals may be re-colonized throughout their lifetime.

### **Virulence Factors**

The pneumococcus expresses several virulence factors that promote its survival and growth in the human host. The nasopharynx is the site of evolutionary pressure for *S. pneumoniae*; therefore, these factors generally promote colonization and persistence, usually by mechanisms of immune

evasion. However, they can also exacerbate disease processes, although disease in the host is relatively rare and has no evolutionary benefit for the bacterium (276).

### *Capsule*

The capsular polysaccharide (Fig. 1.1), commonly referred to simply as 'capsule,' is comprised of long alternating sugar chains that are covalently linked to the bacterial cell wall (256). This thick, sometimes mucoid (284), 'sugar coating' functions as a protective barrier between the bacterial cell and the environment of the upper respiratory tract and is critical for successful colonization of the nasopharynx (161).

Pneumococci of the same strain vary in the amount of capsule that they express, a phenomenon known as phase variation (138, 277). Bacteria that express high levels of capsule are called 'opaque,' a reference to their colony morphology, whereas variants with a low abundance of capsule are deemed 'transparent.' High levels of capsule expression aid in evasion of the host immune system by preventing complement deposition on the bacterial cell surface (119) and opsonophagocytic killing by phagocytes (137). Opaque variants are favored during invasive disease (138), while transparent variants are favored during colonization (277). Capsule can also prevent the action of antibiotics that target pneumococcal cell wall enzymes, presumably by blocking the ability of the antibiotic to penetrate to the cell surface (78).

There are over 90 known capsule types (106), which define the

serotypes of pneumococci. The majority of capsules are negatively charged (130), a feature that allows the pneumococcus to repel the negatively charged mucus layer of the respiratory tract and prevents its clearance by mucociliary beating (197). Some serotypes are more commonly associated with invasive disease and differences in capsule type may contribute to the site of infection (126) and overall prevalence in the population (275), though this remains poorly characterized.

### *Pneumolysin*

The pneumococcus expresses a single pore-forming toxin named pneumolysin, which is a member of the cholesterol dependent cytolysin (CDC) family. Pneumolysin is expressed as a 53-kDa monomer that oligomerizes to form pores in cholesterol containing membranes (185, 270). These pores can be up to 30 nm in diameter (144) and at high concentrations can cause eukaryotic cell lysis (245). The CDC toxin family spans more than 20 bacterial species and all CDC toxins have a highly conserved tryptophan-rich undecapeptide region, which is critical for toxin insertion into host membranes (235, 265). In the case of pneumolysin, a single residue mutation in the undecapeptide results in near complete loss of toxin function (144), though binding to cholesterol occurs through residues outside of the undecapeptide region (73).

Pneumolysin is unique among the CDC toxin family because it lacks an N-terminal secretion signal sequence (270). It is known to be associated with

the bacterial cell wall (218, 219), though the exact mechanism of its release is still not well defined. It was originally believed that pneumolysin secretion was dependent on bacterial autolysis (181). However, pneumococcal strains lacking LytA – the autolytic amidase – are still able to secrete pneumolysin (15). Pneumococcal degradation by the host enzyme lysozyme, which hydrolyzes the glycan backbone of peptidoglycan, can cause the release of pneumolysin in broth culture (60).

Nearly all clinically isolated strains of pneumococci express pneumolysin (131), underscoring its important role in disease, as well as its evolutionary importance to the bacterium. Pneumolysin induces inflammation at the mucosal surface, though how this contributes to the organisms' virulence appears to vary by site of infection or pneumococcal strain (126, 205). Pneumolysin-deficient strains are highly attenuated in multiple disease models (22, 23, 31, 236). Somewhat paradoxically, pneumolysin does not promote the establishment of nasopharyngeal colonization (237) and pneumolysin expression drives host clearance of colonization (171, 267). A mutated strain containing a full deletion of the pneumolysin gene has prolonged carriage in the nasopharynx compared to the isogenic wild type strain (171, 267) and exactly how pneumolysin contributes to the persistence of *S. pneumoniae* in the host or the population remains an open question.



### *Other Virulence Factors*

Although capsule is the immunodominant pneumococcal antigen, the pneumococcal surface protein A (PspA) can elicit cross-reactive antibodies (33). Vaccination with PspA can confer protection against pneumococcal colonization (204), pneumonia (34), and sepsis (255) in murine models. PspA is under active investigation as a potential vaccine target (194) because it is abundantly expressed in all pneumococcal strains and could offer broader strain coverage than is currently available. PspA prevents the deposition of complement onto the bacterial cell surface (226, 227, 264) by competitively inhibiting the binding of C-reactive protein (CRP) (190). Phosphocholine (ChoP), the moiety that is targeted by CRP, anchors the choline binding proteins, including PspA, to the bacterial cell surface (81, 288). Prevention of CRP binding promotes virulence in sepsis models by inhibiting the alternative pathway of complement activation (227, 264). PspA also binds lactoferrin (99, 102), a glycoprotein that sequesters iron, as well as apolactoferrin (242), an iron-free form of lactoferrin. Apolactoferrin has bactericidal properties and binding by PspA promotes pneumococcal survival (242). It is speculated that PspA-mediated interactions with lactoferrin promote iron scavenging by *S. pneumoniae*, though whether this contributes to bacterial growth *in vivo* has not been resolved.

Similar to many other bacterial pathogens and residents of the mucosal flora (32, 85, 86, 143, 188, 214, 215, 217), *S. pneumoniae* expresses a protease that specifically cleaves human immunoglobulin A1

(IgA1) (271). Evasion of this host immunoglobulin in the upper respiratory tract is critical for bacterial persistence, as IgA1 comprises over 90% of the mucosal IgA (140). The pneumococcal IgA1 protease cleaves the hinge region of IgA1, separating the F<sub>C</sub> domain from the antigen-binding Fab fragments. This protease promotes bacterial adherence (278) and prevents complement deposition and opsonophagocytic killing *in vitro* (74), though its specificity for human IgA1 has made *in vivo* animal studies challenging. Recently, however, experiments using mice passively immunized with monoclonal human IgA1 demonstrated that IgA1 protease promotes pneumococcal colonization *in vivo* (124).

Pneumococcal strains express up to three neuraminidases (211), which cleave terminal sialic acid residues from mucins and cell surface glycans in the upper respiratory tract. These neuraminidases promote colonization in the nasopharynx (165, 262) by liberating nutrients for bacterial growth (247) and possibly exposing surface receptors required for adherence to the epithelial surface (210). *S. pneumoniae* also alters its cell wall structure to enhance resistance to the host muramidase lysozyme, which is abundantly expressed on the mucosal surface (49). This modification is energetically costly, but provides an advantage for colonizing organisms (59), although the pneumococcus still remains susceptible to lysozyme degradation by phagocytes (60).

## **Immune recognition of *S. pneumoniae* colonization**

A well-defined murine model of *S. pneumoniae* nasopharyngeal carriage (174), in which unanesthetized mice are intranasally inoculated with pneumococci to restrict bacterial colonization to the upper respiratory tract, has allowed us to further characterize both the host and bacterial factors that contribute to the dynamic process of colonization in the nasopharynx. This model has been validated by experimental human carriage studies with the same pneumococcal strain (173) and has contributed specifically to the elucidation of the host immune cell populations that respond to pneumococcal colonization and lead to eventual bacterial clearance.

### *Neutrophils*

Neutrophils are professional phagocytic cells that are critical in host defense against bacterial and fungal infections. During *S. pneumoniae* colonization, neutrophils are recruited to the upper respiratory tract early in infection, with peak influx generally between one and three days after bacterial inoculation (197, 291). They can be visualized by microscopy in the lumen of the nasopharynx, where they are associated with pneumococci (127). The ability of neutrophils to phagocytose and kill *S. pneumoniae* has been examined *ex vivo* and these studies have demonstrated that bacterial uptake is promoted by opsonization with anti-pneumococcal antibody that facilitates complement deposition (35). *S. pneumoniae* expresses a deoxyribonuclease that confers resistance to killing by neutrophil

extracellular traps (NETs) (20). Instead, bacterial killing by neutrophils is dependent on the action of serine proteases following phagocytosis (251).

Neutrophil numbers in the nasopharynx wane shortly after the establishment of colonization (291). Their influx has minimal impact on bacterial numbers in the upper respiratory tract (171, 291), indicating that they are insufficient to effectively remove the pneumococcus from its niche before type-specific antibody develops. Nevertheless, depletion of neutrophils during colonization with an invasive pneumococcal strain significantly increases mortality in a mouse model (171), suggesting that although they do not promote clearance of nasal carriage, neutrophils are critical for host protection from invasive disease.

### *Macrophages*

During *S. pneumoniae* colonization macrophages are recruited to the upper respiratory tract following initial influx of neutrophils. Their presence peaks by day five post colonization and is maintained for the duration of carriage (60, 291). Depletion of these macrophages through intranasal administration of clodronate liposomes results in higher bacterial burden and a significantly longer duration of colonization (291), demonstrating their importance for effective bacterial clearance.

Host expression of the C-C chemokine receptor type 2 (CCR2), which is present on the surface of inflammatory monocytes, is also required for proper macrophage recruitment. Mice that are deficient in CCR2 expression

(*Ccr2*<sup>-/-</sup>) have significantly lower numbers of nasopharyngeal macrophages over the duration of a colonization event (60), which results in an increased and sustained burden of pneumococci. The C-C chemokine ligand 2 (CCL2) binds CCR2 with the highest affinity of any CCR2 ligand (280) and further investigation showed that CCL2 is produced by macrophages *in vitro* following infection with pneumococci (60). Furthermore, these studies demonstrated that CCL2 production is dependent on bacterial expression of the pore-forming toxin pneumolysin, as well as host expression of the muramidase lysozyme.

Similar to neutrophils, macrophages kill *S. pneumoniae* through phagocytosis and degradation. Although opsonization and complement deposition promote pneumococcal uptake (56), macrophages are able to phagocytose pneumococci in the absence of these components, through scavenger receptor-mediated binding to the bacterial surface. Expression of the scavenger receptor SR-AI/II on alveolar macrophages protects against pneumococcal pneumonia by facilitating bacterial up-take (7). Additionally, the scavenger receptor macrophage receptor with collagenous structure (MARCO) is important in host defense against both pneumonia and *S. pneumoniae* colonization (6, 67).

Following uptake, pneumococci localize to the phagosome (91), which rapidly acidifies (139, 286), resulting in bacterial death (125). Nitric oxide production is important for *S. pneumoniae* killing by macrophages (167), although contributions of additional factors, such as proteases and other

degradative enzymes, has not been reported.

### *Humoral Immune Responses*

The successes of serotherapy and the pneumococcal vaccines clearly demonstrate that high titers of anti-pneumococcal antibodies can protect against disease. Patients with genetic mutations impairing development of antibodies are more susceptible to pneumococcal infection (150), further indicating an important role for antibody-mediated protection, which is believed to be due to increased opsonophagocytic killing by neutrophils (35). Antibodies induced by vaccination can reduce host susceptibility to colonization (54, 180) and studies of experimental human carriage have shown that higher antibody titers correlate with lower pneumococcal numbers in the nasopharynx (173).

Colonization results in the production of both IgA and IgG antibodies that detect the pneumococcal capsule and surface proteins (60, 173, 174, 231, 233). Although antibody can block the acquisition of colonization (232), humoral responses are insufficient to clear pneumococci from an already colonized host (174) and natural immunity to *S. pneumoniae* colonization occurs independent of serotype (156), suggesting that other adaptive immune responses are required to achieve clearance.

### *Cellular Immune Responses*

HIV-positive individuals are more susceptible to pneumococcal disease

and lower CD4<sup>+</sup> cell counts correlate with increased risk of infection (68), indicating that cellular immunity is critical for protection in the human host. Effective clearance of pneumococcal colonization in a murine model requires immunity acquired through CD4<sup>+</sup> T-cells (106, 164), which is attributable specifically to the interleukin-17a (IL-17a) producing Th17 sub-population of CD4<sup>+</sup> T-cells (130, 164, 291). Depletion of these cells during primary colonization prolongs bacterial carriage and dampens host responses to a secondary colonization event (291). These defects correlate with reduced numbers of macrophages and neutrophils during primary and secondary colonization, respectively (291).

### **Innate Immune Detection of *S. pneumoniae***

Hosts, from *Drosophila* to humans, express a finite set of genetically encoded 'pattern recognition receptors' (PRRs) (257). These receptors are highly expressed in cells of the innate immune system and detect conserved bacterial and viral motifs commonly called pathogen associated molecular patterns (PAMPs). PAMPs range from nucleic acids to secretion systems and host recognition of these signatures activates pro-inflammatory signaling cascades that drive innate immune defense. PRRs can broadly be classified by cellular localization and include the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as the cytosolic Nod-like receptors (NLRs), AIM2-like receptors (ALRs), and (RIG)-I-like receptors

(RLRs).

### *Extracellular Receptors*

The TLRs sample the extracellular milieu encountered by host cells. They sense a diversity of bacterial and viral ligands and their activation results in signaling cascades that drive pro-inflammatory cytokine production. All TLRs, except for TLR3, employ myeloid differentiation primary response gene 88 (MYD88) as an adaptor molecule for signaling. Humans with mutations in either MYD88 or interleukin-1 receptor-associated kinase 4 (IRAK-4), a downstream kinase involved in TLR signaling, are acutely susceptible to pneumococcal disease (21, 213). Studies investigating the association of pneumococcal disease with polymorphisms in individual TLRs have yielded inconsistent findings (182, 260), suggesting a possible redundancy with other innate immune pathways that signal through MYD88 and IRAK-4.

Pneumococcal lipoproteins and lipoteichoic acid activate TLR2 both *in vitro* (18, 240, 261) and in the respiratory tract epithelium during *S. pneumoniae* colonization (291). Mice deficient in TLR2 (*Tlr2*<sup>-/-</sup>) have increased susceptibility to invasive pneumococcal disease (19) that may be attributable to disruption of the epithelial barrier (19, 47), which allows greater access to the bloodstream and other sterile sites. *Tlr2*<sup>-/-</sup> mice also have prolonged bacterial carriage and reduced numbers of macrophages recruited to the nasopharynx (291). There are reports that TLR2 is activated



by peptidoglycan from Gram-positive bacteria (241, 287), though the veracity of this observation remains a matter of debate (263).

TLR4, which is classically activated by the lipopolysaccharide (LPS) of Gram-negative bacteria, is also reported to directly recognize the pneumococcal pore-forming toxin pneumolysin and protect against bacterial infection (162, 250). Others (2, 184, 267), however, have observed no effect of TLR4 in *S. pneumoniae* detection and the exact role of this TLR in recognition of pneumococci remains unclear.

*In vitro* studies have shown that TLR9, which recognizes CpG motifs within bacterial DNA (105), is activated upon *S. pneumoniae* infection with live, but not heat-killed, bacteria (184). Further investigations have defined a role for TLR9 activation in early defense against pneumococcal disease *in vivo*, however no effect on colonization has been observed (2).

### *Intracellular Receptors*

NLRs are a family of cytosolic PRRs that are characterized by a nucleotide oligomerization-binding domain (Nod) as well as C-terminal leucine-rich repeats, which are critical for ligand recognition and binding (122, 259). Nod1 and Nod2 detect bacterial peptidoglycan and trigger a signaling cascade that drives pro-inflammatory cytokine production through activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B). Both Nod1 and Nod2 contain N-terminal CARD domains, which facilitate signaling through homotypic CARD-

CARD interactions with their common adaptor protein receptor-interacting protein 2 (RIP2) (24, 120, 121, 203). Nod 1 recognizes the meso-DAP motif of peptidoglycan from Gram-negative bacteria (87), as well a few select Gram-positive organisms, including *Listeria* and *Bacillus* species (45). Nod2 detects the muramyl dipeptide (MDP) found in the cell wall of all bacterial species (88).

Nod2 detection of *S. pneumoniae* peptidoglycan during colonization contributes to macrophage driven bacterial clearance (60). *In vitro* studies have shown Nod2-dependent production of pro-inflammatory cytokines, including interleukin-6 (IL-6) and CCL2, following incubation with pneumococci (60). *In vivo*, host expression of CCR2, the receptor for CCL2, is required for increased macrophage presence in the nasopharynx, as well as subsequent bacterial clearance. In these studies, increased cytokine levels were dependent on host expression of lysozyme and bacterial production of pneumolysin (60), implicating a role for bacterial degradation and toxin release.

Intracellular bacterial pathogens use a variety of strategies to access the cytosol. These mechanisms may provide insight into how pneumococcal products transit into the host cell cytosol (Fig. 1.2). Unlike the model intracellular organisms *Listeria monocytogenes*, which escapes from the phagosome into the cytosol (216), or *Salmonella*, which replicates inside a cellular vacuole (268), *S. pneumoniae* is killed and degraded following phagocytosis. However, all of these microbes activate intracellular host PRRs

(60, 268). *L. monocytogenes* uses its CDC toxin to perforate and destabilize the phagosome membrane, allowing intact bacteria to access the cytosol where they replicate and spread to other cells (216). *Salmonella* uses a type III secretion system to inject effector proteins into the cytosol, which remodel the phagosome to transform it into a hospitable replicative niche. *S. pneumoniae* has no known specialized secretion system, but it does express pneumolysin. It is possible the pneumolysin facilitates transit of bacterial components across the phagosome membrane, however, the exact mechanism by which pneumococcal cell wall components access the host cell cytosol to activate Nod2 remains unclear.

A subset of NLRs and ALRs trigger a defense mechanism known as inflammasome activation. When this type of defense occurs an inflammasome complex, comprised of receptors and adaptor proteins, oligomerizes in the cytosol, causing activation of caspases 1 and 11 (149). The maturation of caspases 1 and 11 into their enzymatically active forms leads to the production of mature interleukin-1 beta (IL-1 $\beta$ ) and interleukin-18 (IL-18), ultimately resulting in the pro-inflammatory death of the host cell (51, 148, 169). Caspase-11 is activated by the acylated lipid A of LPS (98, 137), and therefore detects many intracellular Gram-negative bacterial pathogens (36, 41, 42, 221).

Caspase-1 maturation can result from the activation of a variety of receptors, which detect a diversity of conserved virulence patterns including cytosolic flagellin (4, 83, 178), DNA (77, 114, 192), bacterial secretion

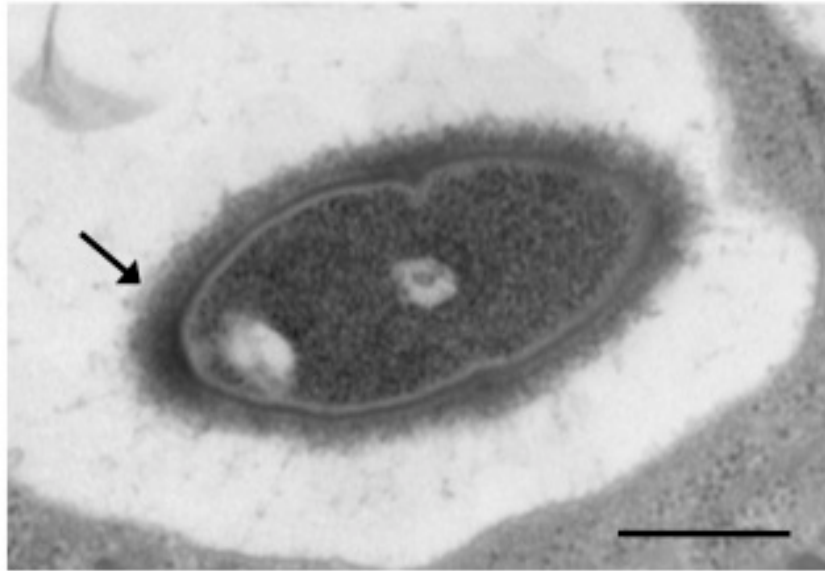
systems (179), and membrane damage (148, 191). *S. pneumoniae* was first characterized to activate the NLRP3 inflammasome (175) and subsequent study revealed that it also activates the AIM2 inflammasome (72). Although they recognize different stimuli, both the NLRP3 and AIM2 inflammasomes assemble through the homotypic interactions of pyrin domains (PYD) at their N-termini with the PYD of the adapter molecule apoptosis-associated speck-like protein containing a CARD (ASC). ASC then recruits procaspase-1 through interactions between its caspase activation and recruitment domain (CARD) and the CARD domain of caspase-1. Recruitment of caspase-1 to this complex results in its activation (238), through what is believed to be an auto-processing mechanism similar to activation of caspases 8 and 9 during intrinsic and extrinsic apoptosis (229), although this has not yet been formally demonstrated.

Activation of the NLRP3 inflammasome by *S. pneumoniae* occurs in multiple cell types including macrophages (72, 282), dendritic cells (175), and neutrophils (133) and is dependent on bacterial expression of pneumolysin. Incubating recombinant pneumolysin with host cells *in vitro* results in NLRP3 dependent pro-inflammatory cell death (175), presumably through action of the toxin at the plasma membrane. In contrast, when phagocytes are infected with live bacteria, NLRP3 and AIM2 mediated cell death is dependent on both bacterial uptake and expression of pneumolysin (72). This suggests that in the context of infection with the entire organism, rather than just its toxin, delivery of inflammasome activating ligands does

not occur across the plasma membrane.

The role of inflammasome activation and subsequent IL-1 $\beta$  secretion during *in vivo* infection has only been partially addressed. Mice deficient in the NLRP3 receptor or ASC adaptor protein had increased bacterial burden and decreased survival in a pneumococcal pneumonia model (72, 175). Similar results were also observed in a model of pneumococcal corneal infection (133). Whether inflammasome activation occurs during pneumococcal colonization and promotes host defense remains to be explored. Given that the pneumococcus activates other cytosolic sensing systems and carriage induces inflammation, it is plausible that IL-1 $\beta$  secretion occurs during colonization and may contribute to eventual bacterial clearance.

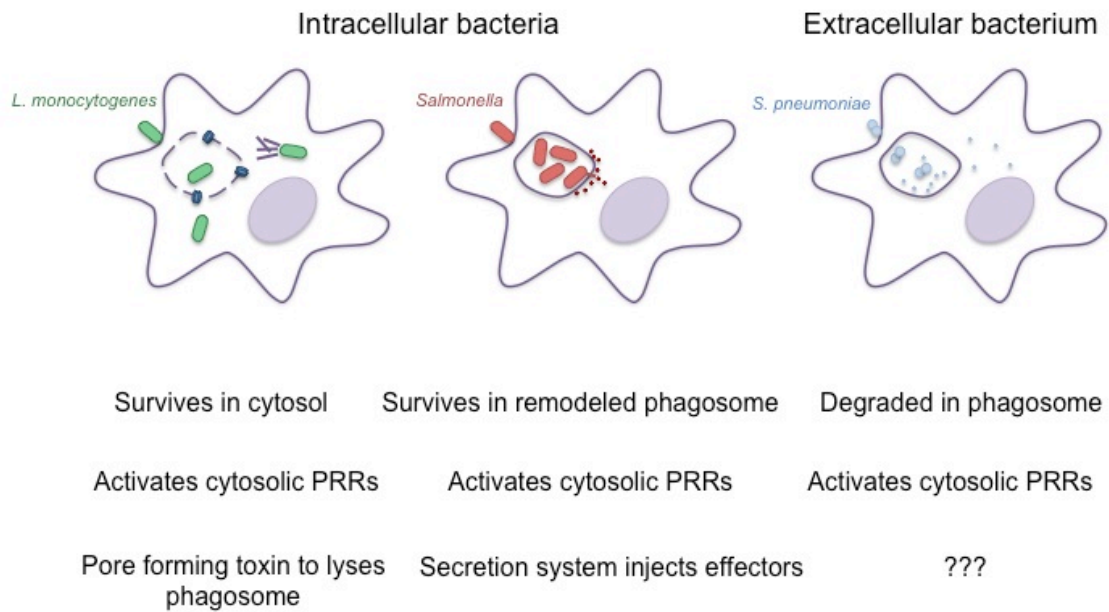
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**Figure 1.1. *Streptococcus pneumoniae* morphology.**

(A) Electron micrograph of the type 23F *S. pneumoniae* strain used in these studies. Black arrow indicates the capsular polysaccharide ('capsule'). Scale bar represents 500 nm.

A



**Figure 1.2. Bacterial strategies to access the cytosol.**

(A) The intracellular bacterial pathogens *Listeria monocytogenes* and *Salmonella* use pore-forming toxins and secretion systems respectively, to gain access to the host cell cytosol to promote their survival. *S. pneumoniae*, in contrast, is killed and degraded, although it activate cytosolic pattern recognition receptors, suggesting cytosolic access occurs during infection.

## **Aims of Dissertation**

The work presented in this dissertation was designed to address the following aims:

- 1) To determine the mechanism by which *S. pneumoniae* products access the host cell cytosol during infection of macrophages

Previous studies from our lab have defined a critical role for macrophages in the clearance of pneumococci from the host nasopharynx. This macrophage influx is dependent on Nod2 recognition of intracellular pneumococcal peptidoglycan, which triggers the expression and secretion of the macrophage chemokine CCL2. Production of CCL2 by macrophages *in vitro* is dependent on bacterial expression of the pneumococcal pore-forming toxin, pneumolysin. Pneumolysin can be released following bacterial lysis by the host muramidase lysozyme. Together these data suggest that cytosolic access occurs following phagocytosis and lysozyme-mediated bacterial degradation that releases pneumolysin, allowing transit of pneumococcal components across the phagosome membrane. Although signaling data support this hypothesis, quantification of pneumococcal components in the host cell cytosol remains to be formally demonstrated.

To assess the mechanism of cytosolic access of pneumococcal components we adapted a detergent-based cellular fractionation assay to measure levels of capsular polysaccharide in the host cell cytosol following infection. This allowed us to interrogate both the host and bacterial factors that contribute to intracellular detection of *S. pneumoniae*.



2) To determine the consequences of cytosolic access on *S. pneumoniae* nasopharyngeal colonization

*In vitro* studies from other investigators have demonstrated that infection of phagocytes with *S. pneumoniae* results in activation of cytosolic innate immune sensors other than Nod2, including multiple receptors involved in inflammasome signaling. A protective role for both inflammasome components and IL-1 $\beta$ , the pro-inflammatory cytokine produced during inflammasome activation, against pneumococcal disease has already been defined. However, the contribution of these cytokines to pneumococcal colonization and the host response to carriage has not been investigated.

To determine the possible effects of inflammasome-derived cytokines on pneumococcal colonization, we intranasally inoculated type 1 IL-1 receptor-deficient mice (*Il1r1*<sup>-/-</sup>) and monitored both bacterial density and host cell infiltrate in the nasopharynx. We further employed quantitative RT-PCR to characterize the chemokine profile of colonized *Il1r1*<sup>-/-</sup> mice, which may contribute to altered host responses during *S. pneumoniae* carriage.

## **CHAPTER II**

**Degradation products of the extracellular pathogen**

***Streptococcus pneumoniae* access the cytosol**

**via its pore-forming toxin**

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## **Abstract**

*Streptococcus pneumoniae* is a leading pathogen with an extracellular lifestyle, however, it is detected by cytosolic surveillance systems of macrophages. The innate immune response that follows cytosolic sensing of cell wall components results in recruitment of additional macrophages, which subsequently clear colonizing organisms from host airways. In this study, we monitored cytosolic access by following the transit of the abundant bacterial surface component capsular polysaccharide, which is linked to the cell wall. Using confocal and electron microscopy, we characterized the location of cell wall components in murine macrophages, and found that they are distributed outside of membrane-bound organelles. Quantification of capsular polysaccharide through cellular fractionation demonstrated that cytosolic access of bacterial cell wall components is dependent on phagocytosis, bacterial sensitivity to the host degradative enzyme lysozyme, and release of the pore-forming toxin pneumolysin. Activation of p38 MAPK signaling is important for limiting access to the cytosol, however ultimately these are catastrophic events for both the bacteria and the macrophage, which undergoes cell death. Our results show how expression of a pore-forming toxin ensures the death of phagocytes that take up the organism, although cytosolic sensing results in innate immune detection that eventually allows for successful host defense. These findings provide an example of how cytosolic access applies to an extracellular microbe and contributes to its pathogenesis.

## **Importance**

*Streptococcus pneumoniae* is a bacterial pathogen that is a leading cause of pneumonia. Pneumococcal disease is preceded by colonization of the nasopharynx, which lasts several weeks before being cleared by the host's immune system. Although *S. pneumoniae* is an extracellular microbe, intracellular detection of pneumococcal components is critical for bacterial clearance. In this study, we show that following bacterial uptake and degradation by phagocytes, pneumococcal products access the host cell cytosol via its pore-forming toxin. This phenomenon of cytosolic access results in phagocyte death and may serve to combat the host cells responsible for clearing the organism. Our results provide an example of how intracellular access and subsequent immune detection occurs during infection with an extracellular pathogen.

## Introduction

Cytosolic detection of pathogen associated molecular patterns is a key event in host discrimination between commensal and pathogenic microbes. While cytosolic access is critical for the pathogenesis of intracellular bacteria, access to the cytosolic compartment by bacteria with an extracellular lifestyle remains poorly understood. One such pathogen that has been shown to activate cytosolic sensing is *Streptococcus pneumoniae*, a Gram-positive bacterium that serially colonizes the human upper respiratory tract (URT) and is a leading cause of bacterial pneumonia (202). Colonization of the upper airway precedes invasive pneumococcal disease (29) and is normally cleared by the host immune response within several weeks (173). A murine model of *S. pneumoniae* colonization has demonstrated that clearance of pneumococcal colonization requires a sustained presence of macrophages in the URT (291), similar to the observation that alveolar macrophages are critical for host defense in the lower respiratory tract (142).

The macrophage driven clearance of colonizing pneumococci is dependent on host expression of the cytosolic Nod-like receptor Nod2 (60). Recognition of the cell wall component peptidoglycan by Nod2 activates NF- $\kappa$ B signaling (88, 203, 259), resulting in expression of pro-inflammatory cytokines, including the monocyte chemoattractant protein CCL2 (or MCP-1). Macrophage recruitment to the URT, as well as subsequent bacterial clearance, is dependent on production of CCL2 *in vivo* and *in vitro* requires bacterial expression of the pneumococcal pore-forming toxin, pneumolysin

(60).

Pneumolysin, a CDC toxin, is expressed as a monomer that oligomerizes to form a pore up to 30 nm in diameter in cholesterol containing membranes (144). Pneumolysin is unique among the CDC family of toxins in that it lacks an N-terminal secretion signal sequence (270), but bacterial degradation by the muramidase lysozyme can cause its release in broth culture (59). Host expression of lysozyme is also required for clearance of pneumococcal colonization (60), suggesting that release of pneumolysin following lysozyme degradation allows peptidoglycan to access the host cell cytosol, where it is sensed by Nod2, eventually resulting in CCL2 production and clearance of colonization. Pneumolysin has been shown to facilitate cytosolic access of cell wall from other bacterial species in epithelial cells for sensing by Nod1 (222), though the mechanism by which Nod2 activation occurs in phagocytes remains to be determined.

Here we show that pneumococcal cell wall associated components escape into the cytosol of macrophages following phagocytosis and degradation. Access to the cytosol is mediated by the pore-forming toxin pneumolysin and is dependent on the ability of pneumolysin to bind host cell membranes. While the host cell has defenses to limit the amount of bacterial products that escape the phagosome, cytosolic access ultimately leads to the pro-inflammatory death of the macrophage.

## Results

### *Pneumococcal components access the cytosol of macrophages*

The process of cytosolic access was monitored by following the transit of the bacterial surface component capsular polysaccharide (CPS), which is covalently linked to the cell wall (256). CPS was chosen as a proxy for cell wall because it is abundant, long lived in the host cell, and easily detectable. To visualize pneumococcal fragments we incubated murine bone marrow derived macrophages (BMMs) with *S. pneumoniae* and, following phagocytosis and degradation, conducted immunofluorescence staining for the pneumococcal CPS and the phagolysosome marker, LAMP1. By confocal microscopy, we observed CPS staining within LAMP1-positive vesicles (Fig. 2.1A, white open arrow), as well as smaller foci of CPS that do not co-localize with the phagolysosome marker (Fig. 2.1A, white closed arrows). To address whether these fragments were in other membrane-bound organelles, we conducted immuno-gold labeling and electron microscopy of BMMs incubated with pneumococci. We observed immuno-gold staining for CPS both within the phagosome and outside of any membrane-bound compartment by 45 minutes after infection (Fig. 2.1C, red arrows). At three hours post-infection the bacteria appeared fully degraded, but gold labeling of CPS was still visible both within and outside the phagosome (Fig. 2.2). Uninfected macrophages had no detectable immuno-gold labeling for CPS (data not shown). These results suggest that pneumococcal products are present in the host cell cytosol following bacterial degradation by BMMs.

The amount of pneumococcal products in the cytosol was more precisely quantified by adaptation of a detergent based sub-cellular fractionation assay. Following infection with *S. pneumoniae*, BMMs were permeabilized with detergents to lyse either all host cell membranes or selectively lyse the plasma membrane. The resulting supernatants were ultracentrifuged to generate purified fractions representing either the cytosol or whole cell contents. The purity of these fractions was validated by western blotting for proteins present in either the cytosol (LDH) or lysosome (Cathepsin B) (Fig. 2.3A), and the amount of pneumococcal CPS in each fraction was quantified by ELISA. The percentage of CPS present in the cytosol fractions was calculated as a proportion of the CPS measured in the whole cell fractions and was significantly higher than background levels of contamination, which were determined by ELISA for the lysosome protein Cathepsin B (Fig. 2.3B). A *S. pneumoniae* strain lacking two cell wall modifying enzymes, making it hypersensitive to degradation by the host enzyme lysozyme (*pgdA-/adr-*) had significantly higher amounts of CPS in the cytosol fraction compared to a wild type (WT) pneumococcal strain (Fig. 2.3B), though no difference in total bacterial uptake was observed between the two strains, as measured by CPS in the whole cell fractions (Fig. 2.3C). In a gentamicin protection assay, both the WT and the *pgdA-/adr-* strains were killed over a 30 minute infection, though this process was more rapid for the *pgdA-/adr-* mutant (Fig. 2.3D). BMM killing of the *pgdA-/adr-* mutant strain was largely lysozyme dependent (Fig. 2.3E), correlating with the lack



of intracellular signaling previously observed in lysozyme deficient BMMs (60). Since the *pgdA-/adr-* strain had significantly higher levels of CPS present in the cytosol compared to the WT strain, unless otherwise specified, subsequent experiments used this lysozyme sensitive *S. pneumoniae* mutant in order to interrogate the host and bacterial factors mediating access of pneumococcal cell wall products to the cytosol.

*Access to the cytosol is dependent on phagocytosis and pneumolysin*

To address the role of bacterial uptake in cytosolic access of pneumococcal cell wall components, we treated BMMs with cytochalasin D (CytD), an inhibitor of actin polymerization, to block phagocytosis. Following infection with *S. pneumoniae* (Spn), the presence of CPS in the whole cell fractions of host cells was blocked by incubation with CytD (Fig. 2.4A). The presence of CPS in the cytosol fractions upon infection with pneumococci was similarly dependent on bacterial uptake (Fig. 2.4B). These results demonstrate that cytosolic access of pneumococcal CPS requires phagocytosis.

The pneumococcal pore-forming toxin, pneumolysin, is required for activation of cytosolic host signaling pathways (60). The role of pneumolysin in cytosolic access of pneumococcal cell wall components was assessed by quantifying the amount of capsule present in the cytosol of BMMs infected with a lysozyme sensitive *S. pneumoniae* strain containing an unmarked, complete in-frame deletion of the pneumolysin gene (*ply-*). The absence of

pneumolysin had no effect on the ability of host cells to take up pneumococci, as seen by similar levels of CPS in the whole cell fractions (Fig. 2.5A). However, compared to a bacterial strain expressing WT pneumolysin, the *ply*<sup>-</sup> mutant had significantly less CPS present in the host cell cytosol (Fig. 2.5B). The loss of pneumolysin expression had no effect on intracellular bacterial killing (Fig. 2.5C). A pneumococcal strain expressing a pneumolysin toxoid, containing alanine mutations in the two residues that bind cholesterol (*ply*<sub>TL-AA</sub>) (73), which is deficient in pore-formation as assessed by hemolysis assay (Fig. 2.6A), but has no defect in amount of pneumolysin protein expressed (Fig. 2.6B), similarly had significantly reduced levels of CPS in the cytosol fraction (Fig. 2.5B). Reintroduction of the pneumolysin gene to correct the mutation (*ply*<sup>+</sup>) fully restored the presence of CPS in the host cell cytosol (Fig. 2.5B). Together, these data show that pneumolysin and specifically toxin binding of the host-membrane are required for cytosolic access of pneumococcal cell wall components.

*Cytosolic access is independent of TLR4 and acidification and is attenuated by p38 MAPK activation*

Toll-like receptor 4 (TLR4) has been reported to aid in host defense through detection of pneumolysin (162). To determine the role of TLR4 in cytosolic access of pneumococcal products, we differentiated BMMs from C57BL/6 (WT) and *Tlr4*<sup>-/-</sup> mice, infected them with pneumococci, and quantified the presence of CPS by fractionation and ELISA. We observed no

difference in the amount of total bacterial capsule in the *Tlr4*<sup>-/-</sup> macrophages compared to WT macrophages (Fig. 2.7A) and there was no significant difference in the amount of CPS in the cytosolic fractions between WT and *Tlr4*<sup>-/-</sup> macrophages (Fig. 2.7B). Cytosolic access, therefore, occurs independently of TLR4 activation.

Acidification of the phagosome occurs rapidly following bacterial uptake. Whether phagosome acidification is required for bacterial products to escape into the cytosol was ascertained by incubating BMMs with bafilomycin A (BAF), a specific inhibitor of the vacuolar ATPase. The ability of BAF to block acidification was validated by western blotting for a lysosomal protease which matures only at low pH (Fig. 2.8). Measurements of CPS showed no difference in total bacterial uptake (Fig. 2.7C) or the amount of capsule in the cytosol (Fig. 2.7D) between macrophages treated with BAF and untreated BMMs. These results show that cytosolic access of pneumococcal components is independent of phagosome acidification.

p38 mitogen-activated protein kinase (MAPK) is an important factor in host defense against diverse bacterial pore-forming toxins (116, 118, 195) and is activated in a pneumolysin-dependent manner in both epithelial cells (223) and macrophages (58). To investigate whether p38 MAPK detection of pneumolysin affects cytosolic access, we treated BMMs with SB203580, an inhibitor of p38 MAPK activation (MAPKi), before infection with *S. pneumoniae* and sub-cellular fractionation. When p38 MAPK was inhibited in BMMs infected with wild type (WT) *S. pneumoniae*, we observed a significant

increase in the amount of CPS in the cytosol fraction (Fig. 2.9B), but no change in total capsule in the whole-cell fraction (Fig. 2.9A). Infection of MAPKi treated BMMs with the *ply*<sup>-</sup> strain showed no such increase in cytosolic CPS amounts, demonstrating that the hypersensitization observed during p38 MAPK inhibition is dependent on bacterial expression of pneumolysin (Fig. 2.9B). These results suggest that pneumolysin dependent activation of p38 MAPK limits the amount of pneumococcal CPS that accesses the cytosol during infection.

#### *Cytosolic access results in macrophage death*

The final fate of host cells following infection with *S. pneumoniae* was ascertained by incubating BMMs with a lysozyme sensitive (*pgdA*<sup>-</sup>/*adr*<sup>-</sup>) bacterial strain expressing pneumolysin or an isogenic *ply*<sup>-</sup> strain (*pgdA*<sup>-</sup>/*adr*<sup>-</sup>/*ply*<sup>-</sup>), in the presence or absence of CytD, and measuring cytotoxicity by release of lactate dehydrogenase (LDH) at 24 hours after infection. BMMs infected with the pneumolysin-expressing mutant had significantly higher levels of cytotoxicity compared to BMMs infected with the isogenic *ply*<sup>-</sup> strain or those treated with CytD (Fig. 2.10A). These results were also observed upon infection of BMMs with the WT and its isogenic *ply*<sup>-</sup> bacterial strains (Fig. 2.10B). Addition of the p38 MAPK inhibitor prior to infection did not significantly alter macrophage cytotoxicity at 24 hours post-infection (Fig. 2.11). These data show that macrophage death occurs following pneumococcal infection and is dependent on bacterial uptake and expression

of pneumolysin.

## Discussion

Many Gram-positive pathogens express members of the CDC family, or other pore-forming toxins (265). These toxins have diverse functions including translocation of effectors by, for example, streptolysin O of *Streptococcus pyogenes* (160), or access to a replicative niche, as in the case of listeriolysin O (LLO) of *Listeria monocytogenes* (216). Unlike LLO, which allows viable bacteria to access the cytosol of host cells, for *S. pneumoniae*, it is bacterial contents following degradation and killing that transit into the cytosol.

We (60) and others (115) have previously characterized Nod2-dependent intracellular sensing of extracellular pathogens. In this study, we adapted a sub-cellular fractionation assay, coupled with detection of CPS by ELISA, to elucidate the mechanism by which cell wall components of the extracellular bacterium, *S. pneumoniae*, escape into the host cell cytosol. Here we visually and quantitatively demonstrate the transit of pneumococcal products in a manner that is dependent on lysozyme, bacterial uptake, pneumolysin, and specifically the ability of pneumolysin to bind and form pores in host membranes. Our results suggest a model in which *S. pneumoniae* is phagocytosed and degraded in the phagosome by lysozyme, which results in release of the pore-forming toxin pneumolysin and allows transit of bacterial components across the phagosome membrane to the cytosol where they can be detected by intracellular host receptors. Others have recently observed phagosome disruption at a later time-point post-

infection, which is independent of pore-formation by pneumolysin (25), suggesting that there may be more than one mechanism of accessing the host cell cytosol.

Lysozyme is abundantly expressed on both the mucosal surface (49) and within professional phagocytes (75). As a result, mucosal pathogens, including *S. pneumoniae* and *L. monocytogenes*, modify their peptidoglycan to confer increased resistance to this degradative enzyme (59, 220). However, these modifications confer only partial resistance because, complementary to previously observed *in vitro* data (59), pneumococcal killing by macrophages *ex vivo* is dependent on lysozyme, and modification to the bacterial cell wall delays, but does not block, bacterial killing. Our observation that cytosolic access does not require acidification of the phagosome is consistent with lysozyme-dependent pneumococcal degradation, which can occur in broth culture at a neutral pH (59).

Previous work has characterized a role for pneumolysin in the transit of other bacterial species' peptidoglycan across the plasma membrane of epithelial cells (222). While it is possible that lysed extracellular pneumococci could access the host cytosol in a similar fashion, our finding that access to the cytosol in professional phagocytes requires bacterial uptake and degradation suggests that the transit of pneumococcal cell wall components we observe occurs across the phagosome membrane, rather than the plasma membrane. Although we have not directly visualized bacterial contents passing through the pore made by pneumolysin, our results demonstrate

that the presence of pneumococcal products in the cytosol requires both toxin expression and function.

P38 MAPK senses a diversity of bacterial toxins including proaerolysin (116) and streptolysin O (223), a cytolysin in the same toxin family as pneumolysin, and plays an important role in host defense. Activation of p38 MAPK occurs rapidly upon incubation of *S. pneumoniae* with macrophages (58), making it likely that initial sensing occurs at the plasma membrane prior to phagocytosis. Furthermore, purified pneumolysin is able to activate p38 MAPK (223) suggesting that detection of toxin activity is independent of host pattern recognition receptors present at the plasma membrane. Our data show that host cell sensing of pneumolysin by p38 MAPK is able to limit the transit of bacterial components to the cytosol, raising the possibility that MAPK activation may attenuate phagosomal damage. Similar observations have been made for *Staphylococcus aureus*  $\alpha$ -toxin, in which p38 MAPK activation is required for cellular recovery, presumably through plasma membrane resealing (118). Lysosomal proteases have been shown to cleave pneumolysin (40), raising the possibility that host proteolysis of this pore-forming toxin may be another mechanism to limit cytosolic access. Ultimately in the case of *S. pneumoniae*, these defenses are insufficient and cytosolic access proves fatal for the host cell.

The observation that host cell death is dependent on bacterial uptake and pneumolysin raises the possibility that accessing the cytosol evolved as a mechanism to ensure death of the host cell that takes up the organism. In



the context of nasopharyngeal colonization, this may allow the pneumococcus to persist by limiting further clearance by the phagocytes that are killed. However, accessing the host cell cytosol results in Nod2-dependent pro-inflammatory signaling, leading to further recruitment of phagocytic cells that aid in bacterial clearance. In addition to Nod2, *S. pneumoniae* is known to activate type I interferon signaling pathways (208) and the cytosolic NLRP3 and AIM2 inflammasomes (72) though strain-to-strain variation has been observed (282). In these studies host cell death was dependent on both pneumolysin expression and bacterial uptake, making it likely that the cell death observed in our study is a result of inflammasome activation. Toxin-dependent activation of the NLRP3 inflammasome and subsequent phagocyte death has also been described for other extracellular pathogens (97). While inflammasome signaling is important for host defense against pneumococcal pneumonia (72, 128, 282), the role of inflammatory cytokines such as IL-1 $\beta$  in nasopharyngeal colonization of *S. pneumoniae* remains to be defined.

Intracellular bacterial pathogens access the cytosol as part of their lifecycle through use of pore-forming toxins or specialized secretion systems (268). These virulence activities, however, expose pathogen associated molecular patterns to host receptors. Here we show that components of *S. pneumoniae*, a leading extracellular pathogen, access the cytosol via its pore-forming toxin, pneumolysin, following degradation and killing in the phagosome. Our results provide an example for how cytosolic access, and

subsequent intracellular innate immune detection, is relevant to a clinically important extracellular microbe.

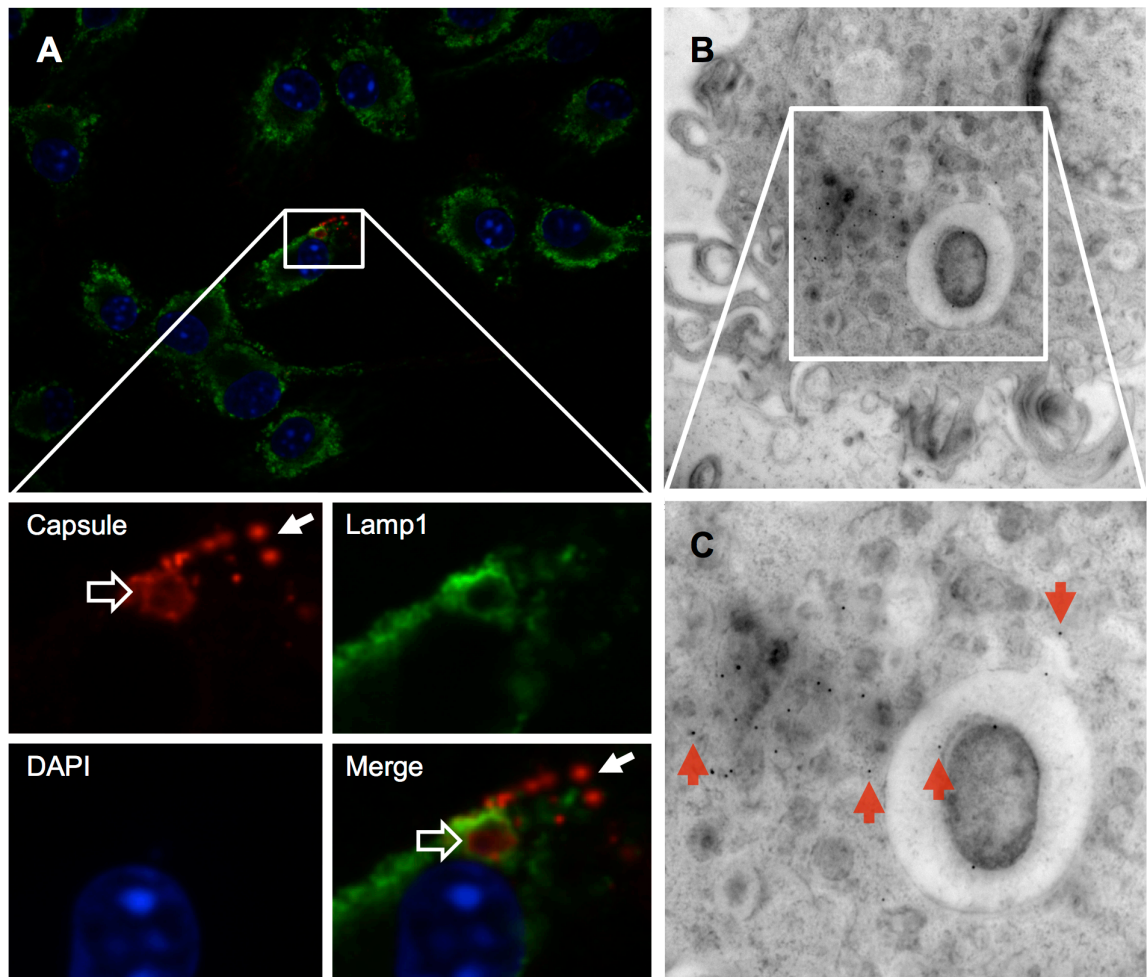
**Acknowledgements**

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We would like to acknowledge the Molecular Pathology and Imaging Core and the Electron Microscopy Resource Laboratory at the University of Pennsylvania.

**Author Contributions**

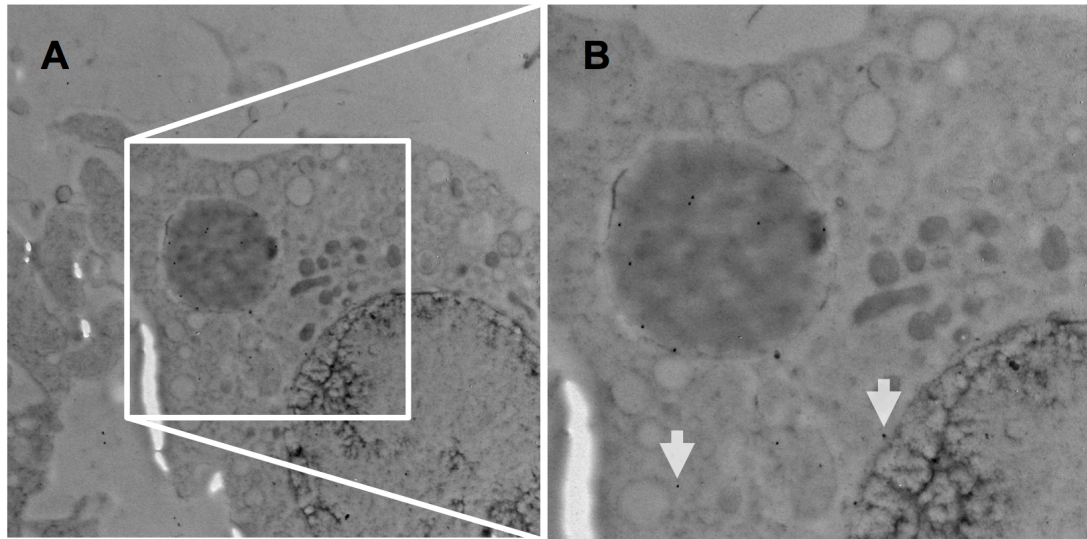
Jamie K. Lemon and Jeffrey N. Weiser were the authors of this work. JKL and JNW designed experiments. JKL conducted experiments, analyzed data, and wrote the manuscript. JKL and JNW edited the manuscript.



**Figure 2.1. Pneumococcal cell wall components access the cytosol of macrophages**

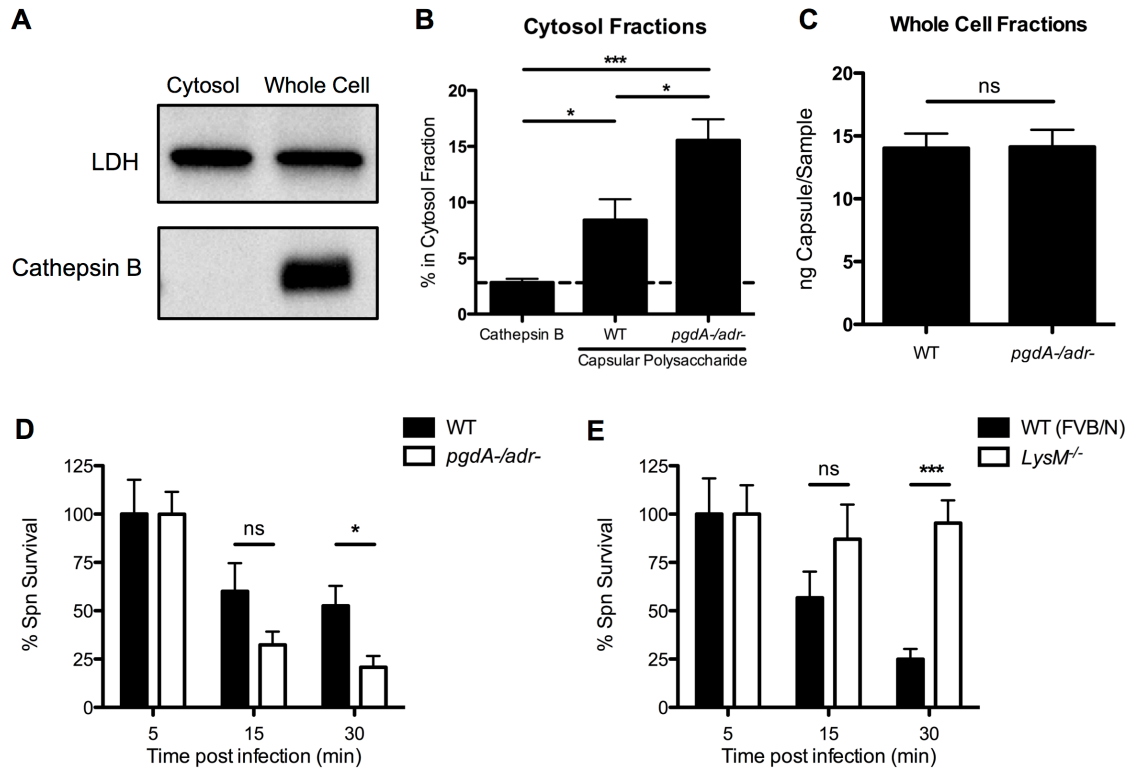
(A) Murine bone marrow derived macrophages (BMMs) were incubated with *S. pneumoniae* for 2.5 hours and stained for immunofluorescence with polyclonal anti-type 23F capsule sera (red, capsule), anti-Lamp1 antibody (green, phagosome), and DAPI (blue, DNA).

(B and C) Electron microscopy of BMMs incubated with *S. pneumoniae* for 45 minutes and immuno-gold labeled with IgG purified from polyclonal anti-type 23F capsule sera. Original magnification, x600 (A), x25,000 (B), and x50,000 (C).



**Figure 2.2. Pneumococcal cell wall components access the cytosol of macrophages**

(A and B) Electron microscopy of BMMs incubated with *S. pneumoniae* for 3 hours and immuno-gold labeled with IgG purified from polyclonal anti-type 23F capsule sera. White arrows indicate gold-particles outside of organelles. Original magnification x15,000 (A) and x50,000 (B).



### Figure 2.3. Quantification of pneumococcal products by cellular fractionation

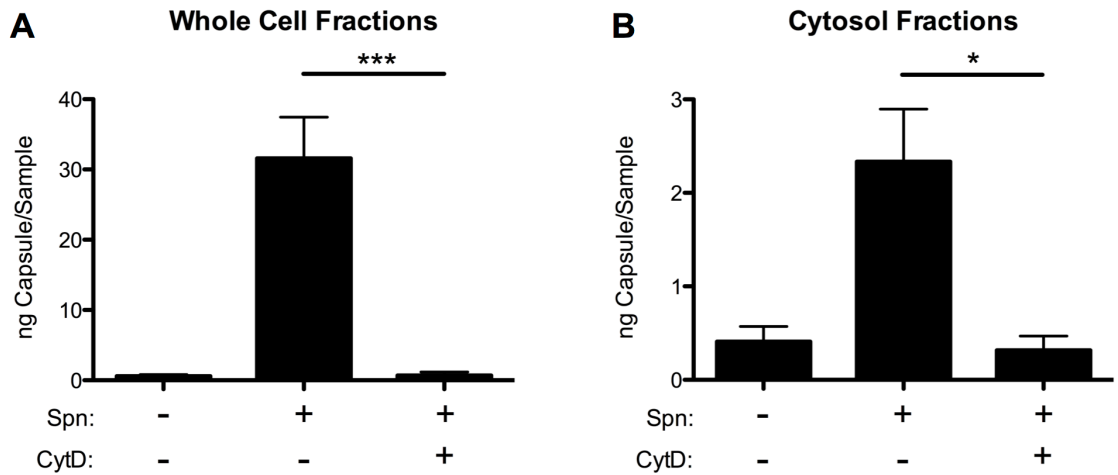
(A) Western blot of sub-cellular fractions from bone marrow-derived macrophages (BMMs) detecting cytosolic (LDH) and lysosome (Cathepsin B) protein.

(B and C) Measurement of contents of cytosol fractions as a proportion of whole cell contents by ELISA. Purity of fractions was measured by Cathepsin B ELISA with dashed line indicating average level of contamination of cytosolic fractions. Quantification of capsular polysaccharide (CPS) in the cytosolic fractions (B) or whole cell fractions (C) of BMMs infected with wild type (WT) or lysozyme hypersensitive (*pgdA-/adr-*) *S. pneumoniae*. Values from 2-5 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \* $p < 0.05$ , \*\*\* $p < 0.001$ ; and ns, not significant.

**Figure 2.3 Continued.**

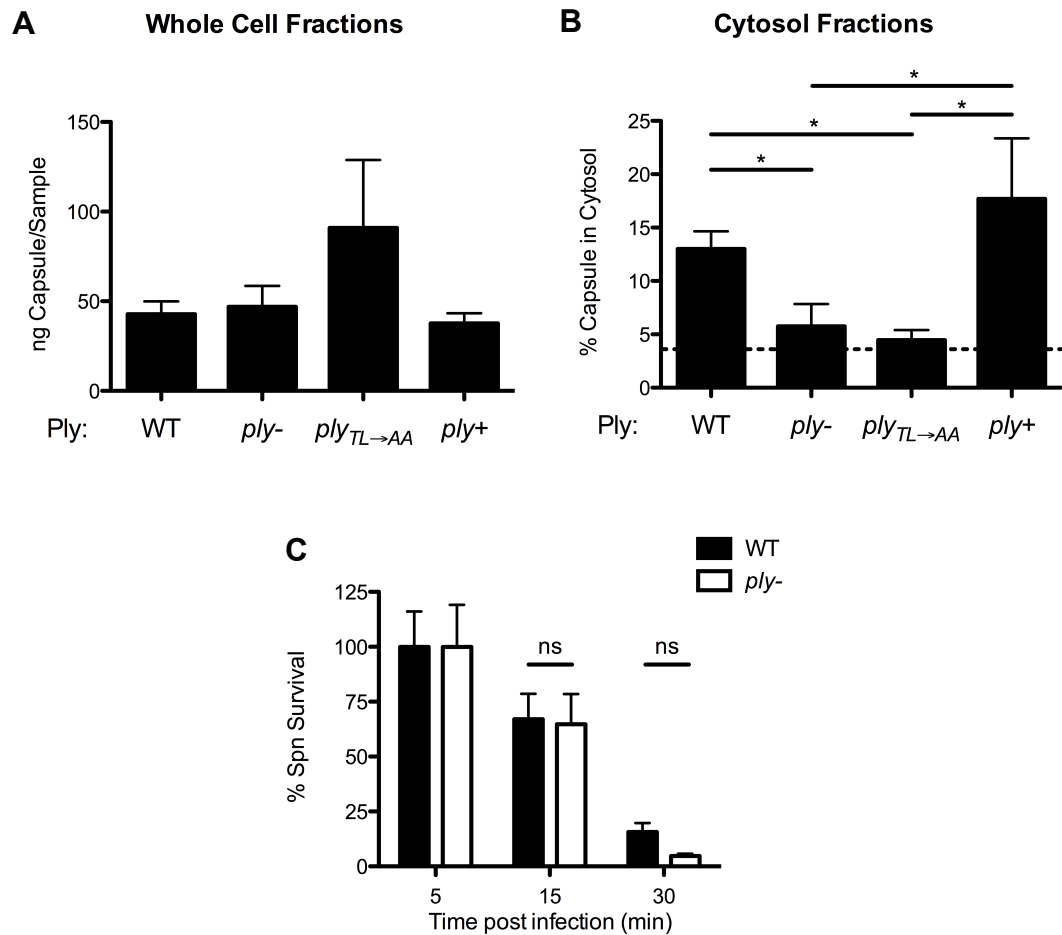
(D and E) Gentamicin protection assay to measure intracellular killing of *S. pneumoniae* (Spn). BMMs were infected with Spn and treated with 300  $\mu$ g/mL gentamicin to kill extracellular bacteria. At the time points indicated, BMMs were lysed with water and serial dilutions plated for CFUs to quantify surviving Spn. C57BL/6 BMMs were infected with WT or *pgdA-/adr-* Spn strains (D) and BMMs from FVB/N or *LysM-/-* mice were infected with the lysozyme sensitive *pgdA-/adr-* Spn strain (E). Percentages were calculated as a proportion of CFUs from the 5 min time point, values from 2 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \* $p < 0.05$ , \*\*\* $p < 0.001$ ; and ns, not significant.





**Figure 2.4. Access to the cytosol is dependent on bacterial uptake**

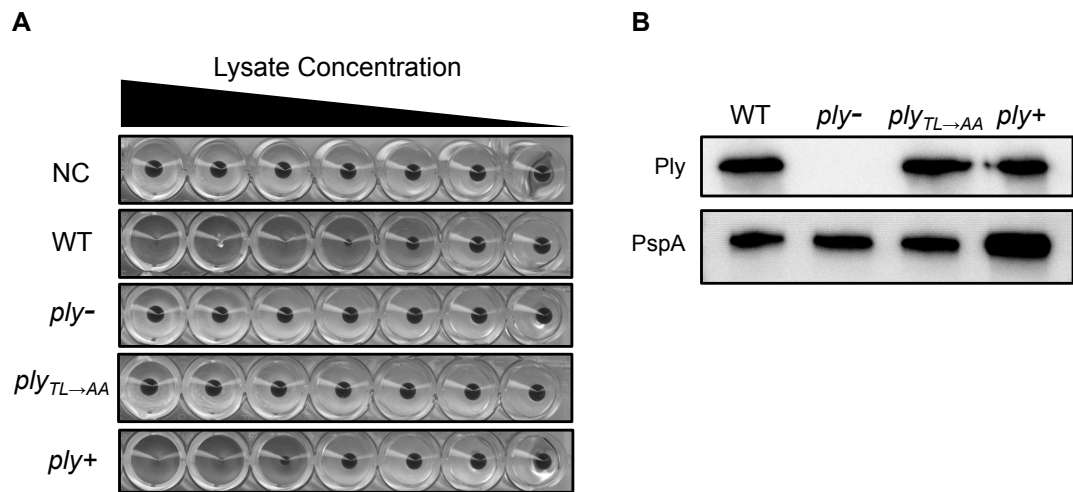
(A and B) Measurement of pneumococcal capsular polysaccharide in whole cell (A) and cytosol (B) fractions of bone marrow derived macrophages (BMMs) by ELISA. Where indicated, BMMs were infected with lysozyme sensitive *S. pneumoniae* (Spn) and treated with 20  $\mu$ M cytochalasin D (CytD) to block phagocytosis. In contrast to other figures, cytosolic fractions are not represented as a proportion of the whole fractions due to nearly undetectable levels of capsule in the CytD treated whole cell fractions. Values from 3 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \* $p < 0.05$ , \*\*\* $p < 0.001$ .



**Figure 2.5. Pneumolysin is required for access to the host cell cytosol**  
 (A and B) Measurement of pneumococcal capsule in whole cell (A) and cytosol (B) fractions of bone marrow derived macrophages (BMMs) by ELISA. Where indicated, BMMs were infected with *S. pneumoniae* strains with wild type (WT) pneumolysin (Ply), complete deletion of the Ply gene (*ply*<sup>-</sup>), a toxoid Ply unable to bind cholesterol (*ply*<sub>TL-AA</sub>), and reintroduction of the full WT Ply gene (*ply*<sup>+</sup>). Dashed line indicates average level of contamination of cytosolic fractions (B). Values from 3-5 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \* $p < 0.05$ .

**Figure 2.5 Continued.**

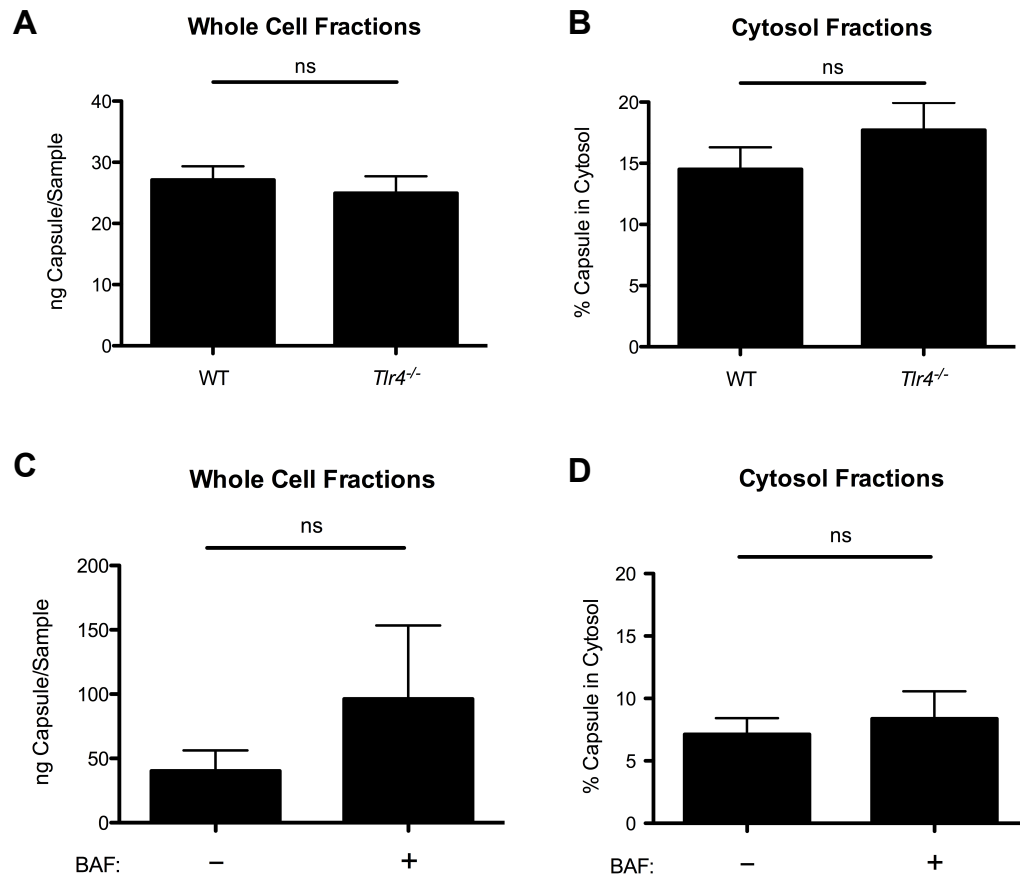
(C) Gentamicin protection assay to measure intracellular killing of *S. pneumoniae* (Spn). BMMs were infected with lysozyme sensitive *S. pneumoniae* strains either deficient for pneumolysin (*ply*-) or expressing wild type (WT) pneumolysin. BMMs were treated with 300  $\mu$ g/mL gentamicin to kill extracellular bacteria and at the time points indicated, were lysed with water and serial dilutions plated for CFUs to quantify surviving Spn. Percentages were calculated as a proportion of CFUs from the 5 min time point, values from 2 independent experiments, Student's t-test, ns, not significant.



**Figure 2.6. Validation of pneumococcal strains expressing pneumolysin mutations**

(A) Hemolysis assay to test for pneumolysin pore-formation. Horse red blood cells (RBC) were incubated with serially diluted lysates from *S. pneumoniae* expressing wild type (WT) pneumolysin, a pneumolysin-deficient strain (*ply*<sup>-</sup>), a toxoid that is unable to bind cholesterol (*ply*<sub>TL→AA</sub>), or a corrected mutant strain (*ply*<sup>+</sup>), for 30 min. Lysis buffer alone was used as a negative control (NC). Unlysed RBCs were pelleted and imaged. Representative images shown.

(B) Western blot for pneumolysin expression. Lysates from lysozyme sensitive *S. pneumoniae* strains were probed for expression of pneumolysin (Ply) or pneumococcal surface protein A (PspA) as a loading control. Representative images shown.

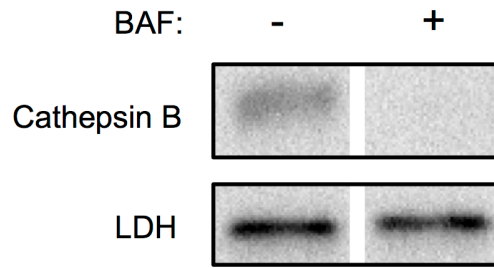


**Figure 2.7. Cytosolic access is independent of TLR4 and acidification**

(A and B) Bone marrow-derived macrophages (BMMs) from C57BL/6 (WT) and *Tlr4*<sup>-/-</sup> mice were infected with *S. pneumoniae* and fractionated. Bacterial capsular polysaccharide (CPS) in the whole cell (A) and cytosol (B) fractions was measured by ELISA.

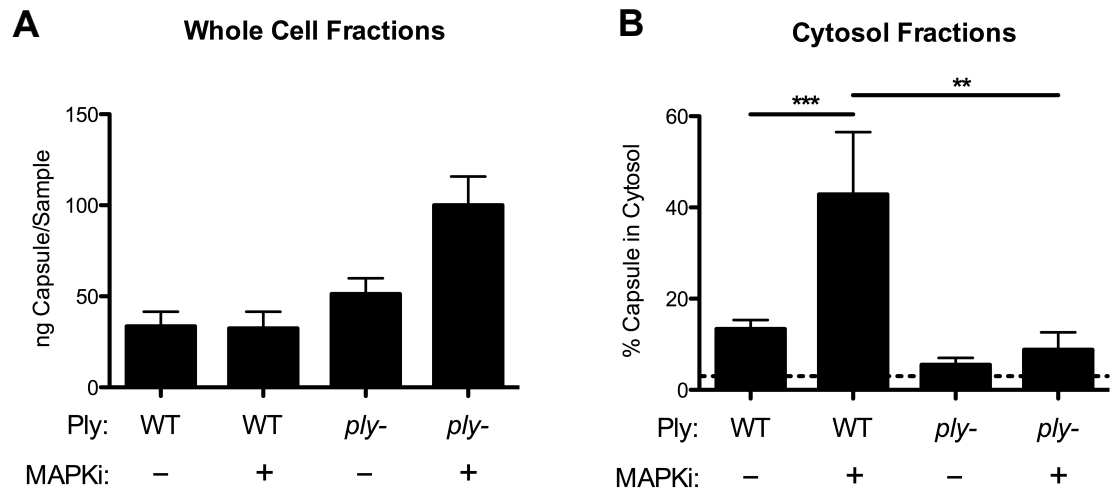
(C and D) BMMs from WT mice were treated with 30 nM bafilomycin A (BAF) prior to infection with *S. pneumoniae* and sub-cellular fractionation. CPS in the whole cell (C) and cytosol (D) fractions was measured by ELISA. Values from 3 independent experiments, error bars represent  $\pm$  SEM, Unpaired t-test, ns, not significant.

**A**



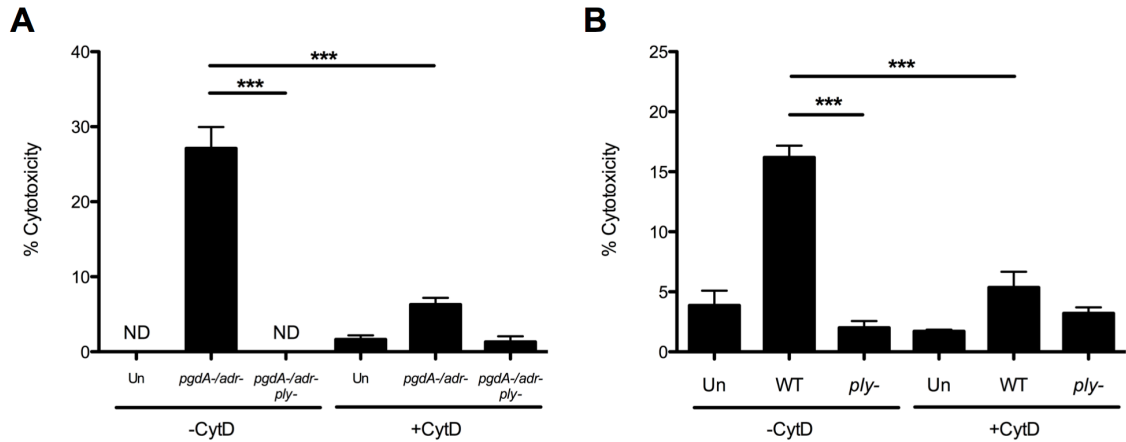
**Figure 2.8. Inhibition of phagosome acidification by bafilomycin A treatment**

(A) Whole cell fractions from *S. pneumoniae* infected macrophages untreated or pre-treated with 30 nM bafilomycin A (BAF) for 1 hour were probed for the lysosomal protein Cathepsin B or the cytosolic protein lactate dehydrogenase (LDH). Images from same blot in non-contiguous lanes.



**Figure 2.9. Cytosolic access is attenuated by p38 MAPK activation**

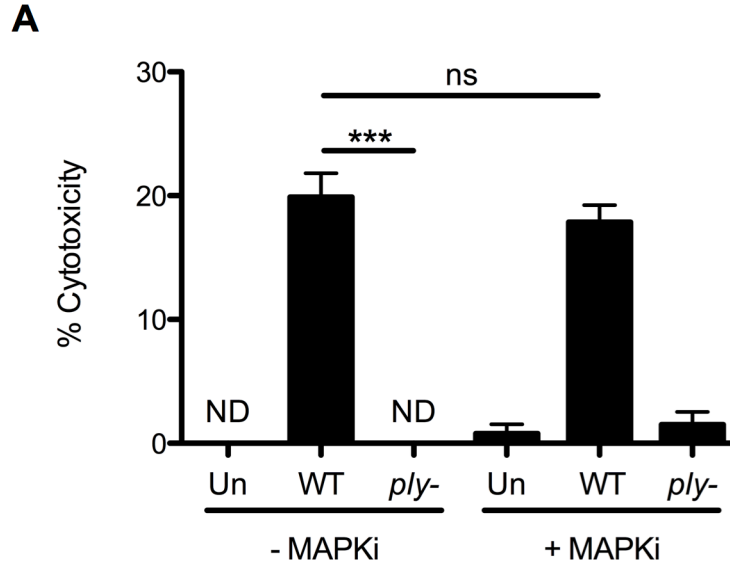
(A and B) BMMs were infected with a *S. pneumoniae* strains expressing pneumolysin (WT) or a pneumolysin-deficient pneumococcal strain (*ply*-). Where indicated, BMMs were treated with SB203580, a specific inhibitor of p38 MAPK (MAPKi). Infected BMMs were fractionated and CPS was quantified by ELISA in the whole cell (A) and cytosol (B) fractions, dashed line indicates average level of contamination of cytosolic fractions. Values from 3-5 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \*\* $p < 0.01$ \*\*\* $p < 0.001$ .



### Figure 2.10. Cytosolic access results in macrophage death

(A-B) Bone marrow-derived macrophages (BMMs) were infected with *S. pneumoniae* strains or left uninfected (Un). Where indicated, BMMs were treated with 20  $\mu$ M cytochalasinD (CytD) to block phagocytosis. Supernatants were collected at 24 hours post infection. Cytotoxicity was measured by release of lactate dehydrogenase (LDH) in supernatants. BMMs were infected with a lysozyme sensitive (*pgdA-/adr-*) and an isogenic pneumolysin deficient (*pgdA-/adr-/ply-*) (A) or wild type (WT) and pneumolysin deficient (*ply-*) strains (B). Values are from 2-4 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \*\*\* $p < 0.001$ , ND, not detected.





**Figure 2.11. Late macrophage death is not affected by prior p38 MAPK inhibition.**

(A) Bone marrow-derived macrophages (BMMs) were infected with wild type (WT) or pneumolysin-deficient (*ply*-) *S. pneumoniae* strains or left uninfected (Un). Where indicated, BMMs were treated with SB203580, a specific inhibitor of p38 MAPK (MAPKi). Supernatants were collected at 24 hours post-infection and cytotoxicity was measured by release of lactate dehydrogenase (LDH). Values are from 2 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \*\*\* $p < 0.001$ , ND, not detected.

## **CHAPTER III**

### **Sensing of IL-1 cytokines during *Streptococcus pneumoniae* colonization contributes to macrophage recruitment and bacterial clearance**

The contents of this chapter were submitted to: *Infection and Immunity*

## Abstract

*Streptococcus pneumoniae*, a leading cause of bacterial disease, is most commonly carried in the human nasopharynx. Colonization induces inflammation that promotes the organism's growth and transmission. This inflammatory response is dependent on intracellular sensing of bacterial components, which access the cytosolic compartment via the pneumococcal pore-forming toxin pneumolysin. *In vitro*, cytosolic access results in cell death that includes release of the pro-inflammatory cytokine IL-1 $\beta$ . IL-1 family cytokines, including IL-1 $\beta$ , are secreted upon activation of inflammasomes, though the role of this activation in the host immune response to pneumococcal carriage is unknown. Using a murine model of pneumococcal nasopharyngeal colonization, we show that mice deficient in the interleukin-1 receptor type 1 (*Il1r1*<sup>-/-</sup>) have reduced numbers of neutrophils early after infection, fewer macrophages later in carriage, and prolonged bacterial colonization. Moreover, intranasal administration of IL-1 $\beta$  promoted clearance. Macrophages are the effectors of clearance and characterization of macrophage chemokines in colonized mice revealed that *Il1r1*<sup>-/-</sup> mice have lower expression of the C-C motif chemokine ligand 6 (CCL6), correlating with reduced macrophage recruitment to the nasopharynx. IL-1 family cytokines are known to promote adaptive immunity. However, we observed no difference in the development of humoral or cellular immunity to pneumococcal colonization between wild type and *Il1r1*<sup>-/-</sup> mice. Our findings show that sensing of IL-1 cytokines during

colonization promotes inflammation without immunity, which may ultimately benefit the pneumococcus.

## Introduction

*Streptococcus pneumoniae* is an opportunistic bacterial pathogen that is responsible for over 1 million deaths annually, mostly in children under the age of five (283). The pneumococcus serially colonizes the mucosal surfaces of the human upper respiratory tract, and carriage of the organism provides the reservoir for all pneumococcal disease (29). Colonization induces airway inflammation that is characterized by a suppurative rhinitis and increased mucus secretion. These secretions promote bacterial growth (247) and inflammation is important for bacterial transmission in a viral co-infection model (244). Human studies have demonstrated that higher bacterial burdens are correlated with a more profound rhinitis (234). However, as a result of this inflammatory response, colonization is normally cleared by the host immune system within several weeks (173).

A well-defined murine model of pneumococcal colonization (174) has elucidated bacterial and host factors that are critical to immune recognition of the pneumococcus, which drives the eventual resolution of the carrier state. Although early colonization triggers the recruitment of neutrophils, these are ineffective at resolving the infection. Clearance of pneumococci from the upper airway over a period of weeks requires a sustained presence of macrophages in the nasopharynx (291). Although *S. pneumoniae* is an extracellular pathogen, this macrophage influx is the result of intracellular innate immune recognition by the cytosolic receptor Nod2 (60). Nod2 detects peptidoglycan (88, 259) that accesses the macrophage cytosol via the

pneumococcal pore-forming toxin, pneumolysin, following phagocytosis and bacterial degradation (151). Nod2 activation results in nuclear factor  $\kappa$ B activation (203) and drives production of pro-inflammatory cytokines, such as CCL2, which contributes to macrophage dependent pneumococcal clearance (60). Cytosolic access, however, is a fatal event for the host cell following bacterial uptake (151). The type of cell death that occurs and its contribution to the host immune response remains unclear.

Our further *in vitro* studies show that macrophage death results in pneumolysin-dependent release of the pro-inflammatory cytokine IL-1 $\beta$ , indicating activation of the inflammasome. Inflammasomes are multi-protein cytosolic complexes that oligomerize through homotypic domain interactions and are comprised of a sensor, which detects bacterial stimuli, and adaptor proteins that recruit procaspase-1. This recruitment drives caspase-1 enzymatic activation, which processes pro-IL-1 $\beta$  and pro-IL-18 into their mature forms and results in a pro-inflammatory cell death known as 'pyroptosis' (51).

The role of inflammasome activation and the production of IL-1 family cytokines in pneumococcal disease has been characterized (72, 128, 133, 175). However, carriage rather than disease is the predominant state for pneumococci in the host. The contribution of pro-inflammatory cell death to immunity against this extracellular pathogen during colonization has not been determined. Here we show that host sensing of IL-1 family cytokines *in vivo* is required for the macrophage presence in the nasopharynx and

clearance of pneumococcal colonization. IL-1 cytokine production in response to the pneumococcus contributes to inflammation in the upper airway, without driving the eventual development of adaptive immune responses.

## Results

### *Pneumococcal infection results in IL-1 family cytokine expression in vitro and in vivo*

We have previously reported that infection of macrophages with a type 23F strain of *S. pneumoniae* results in host cell death, subsequent to bacterial uptake and pneumolysin-dependent cytosolic access of pneumococcal fragments (151). To determine whether pneumolysin-dependent cytosolic access causes pro-inflammatory cytokine release upon *in vitro* infection, we incubated BMMs with the wild type 23F isolate (WT) and 24 hours later assayed for the presence of IL-1 $\beta$  in the cell culture supernatants by Western blotting. We observed the release of mature IL-1 $\beta$  by BMMs infected with WT bacteria, but not in the supernatants of BMMs infected with an isogenic pneumolysin-deficient strain (*ply*-) (Fig. 3.1A). Furthermore, pre-incubation with CytD, an inhibitor of actin polymerization reduced the amount of IL-1 $\beta$  secreted, suggesting that pro-inflammatory cytokine production is dependent on bacterial uptake. Quantification by ELISA showed that release of IL-1 $\beta$  by BMMs was significantly reduced during infection with the *ply*- strain and when phagocytosis was blocked by CytD treatment (Fig. 3.1B). Additionally, enzymatic processing of caspase-1 and production of its active p10 fragment correlated with secretion of IL-1 $\beta$  (Fig. 3.1A). These results show that following infection with a WT pneumococcal strain, BMMs release the pro-inflammatory cytokine IL-1 $\beta$  and that this is dependent on bacterial uptake and expression of the pneumococcal pore-



forming toxin.

To ascertain whether IL-1 family cytokines are expressed *in vivo* during pneumococcal colonization, mice were intranasally inoculated with the WT *S. pneumoniae* strain. Cytokine levels could not be detected because the airway surface fluid is highly diluted in lavages from the upper respiratory tract. Therefore, lysates of the respiratory epithelium were obtained and quantitative RT-PCR (qRT-PCR) performed to assess *Il1a* (Fig. 3.2A) and *Il1b* (Fig. 3.2B) gene expression. We observed that upon colonization with *S. pneumoniae* (Spn) *Il1b* (Fig. 3.2B) was significantly up-regulated compared to mock colonized mice. A much smaller but significant increase in *Il1a* (Fig. 3.2A) expression was observed. Inflammasome components, including pro-IL-1 $\beta$ , are up-regulated upon TLR signaling and this 'priming' provides the first signal required for inflammasome activation (16, 38, 129). Previous studies of inflammasome activation and IL-1 $\beta$  release following pneumococcal infection *in vitro* have characterized TLR2 dependent IL-1 $\beta$  secretion (282). To determine the role of TLR2 in the up-regulation of *Il1a* and *Il1b* gene transcripts during pneumococcal colonization, we inoculated TLR2-deficient (*Tlr2*<sup>-/-</sup>) mice with WT Spn, obtained lysates of the epithelial tissue, and performed qRT-PCR to measure *Il1a* and *Il1b* transcription. We found that *Tlr2*<sup>-/-</sup> mice had no significant up-regulation of *Il1a* (Fig. 3.2C) or *Il1b* (Fig. 3.2D), demonstrating that the increased expression of both genes *in vivo* is dependent on TLR2.

*Sensing of IL-1 cytokines is required for inflammation and macrophage driven bacterial clearance*

The role of IL-1 sensing in early events in pneumococcal colonization was investigated by inoculating wild type (WT) and type 1 IL-1 receptor deficient (*Il1r1*<sup>-/-</sup>) mice with *S. pneumoniae* and obtaining PBS lavages of the upper respiratory tract at 3 days post-infection. We assessed bacterial burden and host immune cell infiltrates by plating for colonizing pneumococci and flow cytometry, respectively. We observed no significant difference in bacterial numbers in the nasopharynx (Fig. 3.3A), however, there were significantly lower numbers of neutrophils in lavages from the *Il1r1*<sup>-/-</sup> mice compared to WT mice (Fig. 3.3B). Macrophage numbers were low, consistent with previous studies (60, 291), and there was no significant difference between the two groups (Fig. 3.3C). These results suggest that *Il1r1*<sup>-/-</sup> mice have a dampened early inflammatory response to *S. pneumoniae* colonization.

To address the role of IL-1 sensing in subsequent clearance of colonization, WT and *Il1r1*<sup>-/-</sup> mice were inoculated with *S. pneumoniae* and bacterial counts and cellular infiltrates were quantified at 14 days post-infection. We observed that WT mice had a significantly lower bacterial load compared to day 3 post-infection (Fig. 3.3A), indicating a partial clearance of pneumococcal colonization. In contrast, *Il1r1*<sup>-/-</sup> mice had no difference in bacterial numbers between days 3 and 14 post-colonization (Fig. 3.3A). Additionally, two weeks after inoculation there was a significantly higher

bacterial burden in the *Il1r1*<sup>-/-</sup> mice compared to WT (Fig. 3.3A). There were similar quantities of neutrophils present in the nasopharynx (Fig. 3.3D) of WT and *Il1r1*<sup>-/-</sup> mice. However, the numbers of macrophages, the effectors of pneumococcal clearance (291), were significantly decreased in the *Il1r1*<sup>-/-</sup> group (Fig. 3.3E). This attenuated clearance and delayed macrophage recruitment resembles previous findings in *Tlr2*<sup>-/-</sup> mice and WT mice colonized with a pneumolysin-deficient mutant (267, 291), both factors implicated in inflammasome activation. From these findings we conclude that sensing by the IL-1 receptor is required for increased macrophage presence in the nasopharynx and clearance of pneumococci.

The contribution of IL-1 $\beta$  to clearance of pneumococci from the nasopharynx was assessed by administration of recombinant cytokine after colonization with a pneumolysin-deficient *S. pneumoniae* strain. This strain was used to minimize endogenous production of cytokine. Following the establishment of colonization, the mice were intranasally dosed with a low (100 ng) or high (200 ng) amount of IL-1 $\beta$  every other day. At 14 days post-infection PBS lavages of the respiratory tract were obtained and bacterial quantity was measured. We observed a significant dose-dependent decrease in bacterial numbers when mice received intranasal IL-1 $\beta$  (Fig. 3.3F). This suggests that IL-1 $\beta$  is sufficient to promote clearance of pneumococcal colonization.

*Il1r1*<sup>-/-</sup> mice have altered macrophage chemokine expression

Previous studies investigating clearance of pneumococcal colonization have characterized an essential role for the host C-C chemokine receptor CCR2 in macrophage recruitment and retention in the nasopharynx (60). CCR2 binds multiple ligands in the C-C motif chemokine family, that act as macrophage attractants (170). To assess the role of IL-1 sensing in macrophage recruitment to the nasopharynx we investigated the expression of several CCR2 ligands during pneumococcal colonization. WT and *Il1r1*<sup>-/-</sup> mice were inoculated with *S. pneumoniae* and at days 3 and 14 post-colonization we obtained lysates of the upper respiratory tract and determined chemokine expression by qRT-PCR. We observed no significant difference between WT and *Il1r1*<sup>-/-</sup> mice in expression of any CCR2 ligand at day 3 post-inoculation (Fig. 3.4A). At day 14 post-infection, when clearance had initiated, *Il1r1*<sup>-/-</sup> mice had significantly lower expression of *Ccl6* compared to WT mice (Fig. 3.4B). These findings demonstrate that *Il1r1*<sup>-/-</sup> mice have an altered macrophage chemokine profile, which includes a reduction in CCL6 expression that correlates with lower macrophage presence and delayed clearance.

*Il1r1*<sup>-/-</sup> mice do not have altered adaptive immunity to the pneumococcus

Although there is a contribution of type-specific antibody to protection induced by either vaccination or prior exposure (55, 163), effective clearance of primary colonization requires Th17-dependent cellular immunity rather than humoral responses (157, 291). IL-1 $\beta$  is a known driver of Th17

responses (46), which contribute to macrophage recruitment during colonization (291). We investigated this potential role of IL-1 sensing in cell-mediated immunity by comparing expression of *Il17a* in WT and *Il1r1*<sup>-/-</sup> colonized mice by qRT-PCR. We observed no significant difference in *Il17a* transcription between the WT and *Il1r1*<sup>-/-</sup> mice (Fig. 3.5A). To address a possible effect on humoral immunity, we measured the geometric mean titer (GMT) of whole anti-pneumococcal serum IgG from WT and *Il1r1*<sup>-/-</sup> colonized mice. Both groups had detectable antibody titers, though there was no significant difference in GMT (Fig. 3.5B). The development of adaptive immunity to the pneumococcus was interrogated using a secondary challenge model. WT and *Il1r1*<sup>-/-</sup> mice were colonized with *S. pneumoniae* and allowed to clear for a period of 8 weeks. The mice were then re-challenged with a marked isogenic strain that could be distinguished from the primary challenge strain and at day 4 post-colonization nasal lavages were obtained and bacterial quantity measured. Compared to the bacterial burden observed at day 3 during primary colonization, WT mice had significantly lower CFUs in the nasopharynx upon secondary challenge (Fig. 3.5C). Similarly, the *Il1r1*<sup>-/-</sup> mice also exhibited a significant drop in bacterial levels upon secondary colonization of an immune host (Fig. 3.5C). Together these data show that signaling through the IL-1 receptor contributes to inflammation without impacting adaptive immune responses to pneumococcal colonization.

## Discussion

Host defense against *S. pneumoniae* requires the activity of professional phagocytes and previous studies have defined a critical role for macrophages in the clearance of colonization (291). This macrophage presence in the nasopharynx is driven by sensing of pneumococcal components that access the host cell cytosol through the action of the pore-forming toxin pneumolysin (60, 151). Cytosolic access results in death of the phagocytes that clear the organism. However, this appears to result in pro-inflammatory cytokine production, mediated by activation of the inflammasome, which further contributes to sustaining the inflammatory response against the colonizing pneumococci.

Pneumococcal strains have been reported to vary in their ability to activate inflammasome signaling (282), however our observation that macrophages activate caspase-1 and secrete IL-1 $\beta$  following pneumococcal infection aligns with previous studies that have reported similar findings in other cell types with different pneumococcal strains (72, 133, 175). These investigations have demonstrated a pneumolysin-dependent activation of both the NLRP3 (133, 175) and AIM2 inflammasomes (72), which sense membrane perturbations and cytosolic DNA respectively. While recombinant pneumolysin alone, presumably acting at the plasma membrane, can activate the NLRP3 inflammasome (175), caspase-1 maturation and IL-1 $\beta$  secretion following infection with whole bacteria requires phagocytosis (72). This suggests that the virulence activity of pneumolysin, which triggers

inflammasome formation, occurs at the phagosome membrane.

The NLRP3 receptor and ASC signaling adaptor protein have both been implicated in defense against pneumococcal pneumonia (72, 175) and corneal infection (133). However, nasopharyngeal colonization is the most common pneumococcal-host interaction and until now the role of inflammasome derived innate immune signaling during carriage has not been addressed. While we have defined a critical role for sensing by the IL-1 receptor during of *S. pneumoniae* colonization, the specific IL-1 receptor agonists that contribute to bacterial clearance remain to be defined. Nevertheless, IL-1 $\beta$  is highly expressed during colonization and is sufficient to reduce *S. pneumoniae* density.

Our observation that sensing of IL-1 family cytokines contributes to inflammation in the nasopharynx following pneumococcal colonization complements previous reports that IL-1 $\beta$  and IL-1 receptor signaling is important for host defense against disease states caused by other mucosal pathogens, including *Staphylococcus aureus* (117) and group B *Streptococcus* (GBS) (27). Both of these organisms trigger IL-1 $\beta$  secretion through toxin-dependent activation of the inflammasome (97, 166), which drives neutrophil activation and pro-inflammatory cytokine production that controls infection (26, 189). The contribution of inflammasome signaling during natural carriage of these organisms remains unexplored. Unlike *S. aureus* or GBS, neutrophil responses are insufficient to clear *S. pneumoniae* during infection (291) and IL-1 $\beta$  appears to contribute to the macrophage

recruitment that promotes reduction in bacterial burden. The requirement for IL-1 sensing in macrophage recruitment during bacterial infection is less well understood. However, IL-1 sensing in the lung during fungal infection is known to enhance expression of both neutrophil and macrophage chemokines (39). Macrophage presence during pneumococcal colonization is dependent on host expression of CCR2, which binds multiple ligands. We observe that *Il1r1*<sup>-/-</sup> mice have diminished macrophage numbers in the nasopharynx two weeks after pneumococcal colonization, which correlates with significantly lower expression of CCL6.

CCL6 is a C-C motif chemokine originally identified in murine bone marrow (206). It acts as a macrophage chemoattractant (147) and is highly expressed in peripheral eosinophils and elicited macrophages (147, 285), as well as lung tissue (109, 132) and epithelial cells of the intestinal mucosa (145). In addition to its chemokine functions, CCL6 may have intrinsic antibacterial properties (145) and over expression of CCL6 in transgenic mice confers protection against bacterial sepsis (48). Multiple studies have observed that in contrast to CCL2, which is expressed early during inflammation, CCL6 induction occurs later and is sustained for several days to over a week (207, 285). These results, from a model of peritonitis, suggest that CCL2 acts in initial macrophage recruitment and that CCL6 sustains macrophage presence. This raises the possibility that IL-1 cytokines, generated as a result of pro-inflammatory macrophage death during pneumococcal colonization, contribute to the chemokine production that



sustains macrophage presence throughout the course of bacterial clearance.

Previous studies have defined an important role for the intracellular receptor Nod2 in innate immune defense against pneumococci (60). However, in these studies, a minimal clearance defect was observed in Nod2-deficient mice, although mice lacking both Nod2 and TLR2 had a significantly higher bacterial burden at a time point when WT mice had no bacterial carriage. Combined with our observation that up-regulation of IL-1 cytokines is dependent on TLR2, this suggests that cytosolic access of pneumococcal components triggers two innate immune sensing pathways, both of which contribute to the orchestrated immune response that clears colonization. Activation of multiple sensing pathways may also be critical for controlling pneumococcal disease. Patients deficient in MYD88 or IRAK-4, adaptor proteins for both TLRs and the IL-1 receptor, are acutely susceptible to recurrent pneumococcal sepsis (21, 50, 146, 212, 213). However, polymorphisms in TLRs alone do not correlate with significant increases in *S. pneumoniae* disease (182), suggesting that sensing by both TLRs and the IL-1 receptor may be critical for host defense.

Although pneumolysin-mediated cytosolic access triggers innate immune responses that eventually clear *S. pneumoniae*, this may still be to the benefit of the bacterium. Cytosolic access results in the death of the phagocytes that clear the organism and generates IL-1 family cytokines that drive inflammation, an important factor in both bacterial growth (247) and transmission (244). Sensing of IL-1 cytokines, however, does not appear to

contribute to the development of an adaptive immune response, suggesting that toxin expression and cytosolic access drives IL-1 cytokine-mediated inflammation that benefits the organism without inducing further immunity that is detrimental to its persistence.

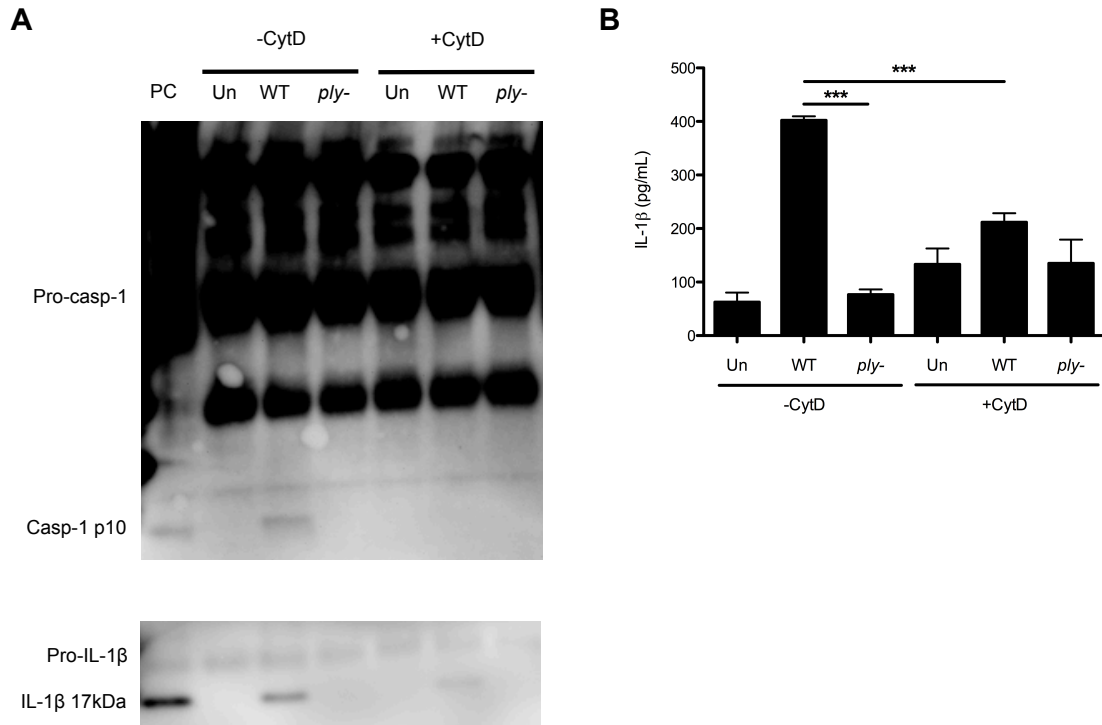
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**Author Contributions**

Jamie K. Lemon, Megan R. Miller, and Jeffrey N. Weiser were the authors of the work presented in this chapter. JKL and JNW designed experiments. JKL and MRM conducted experiments. JKL wrote the manuscript and JKL and JNW edited the manuscript.

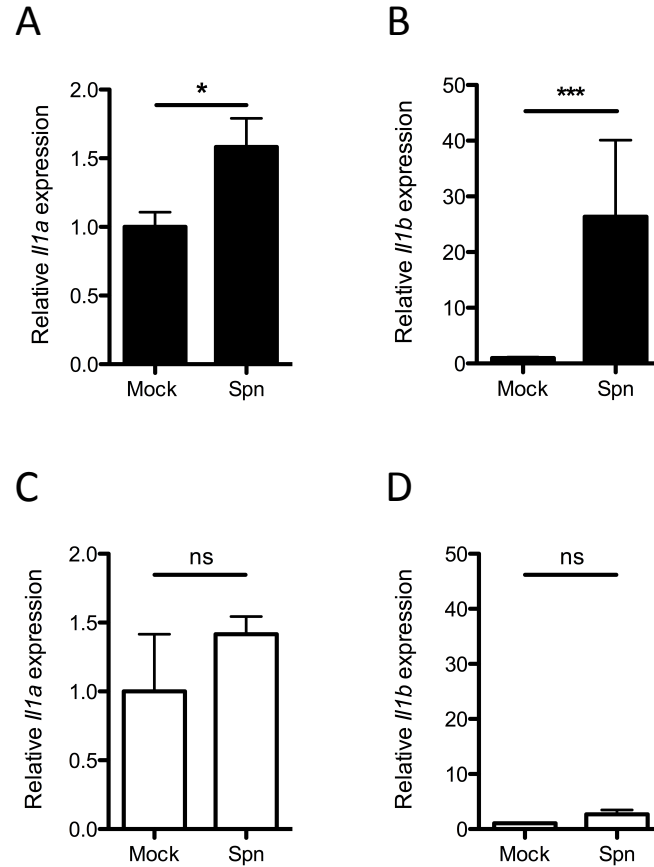


**Figure 3.1. Pneumococcal infection results in IL-1 $\beta$  release *in vitro***

(A and B) Bone marrow-derived macrophages (BMMs) were infected with wild type (WT) or pneumolysin-deficient (*ply*-) *S. pneumoniae* strains or left uninfected (Un). Where indicated, BMMs were treated with 20 $\mu$ M cytochalasin D (CytD) to block phagocytosis. Supernatants were collected at 24 hours post infection.

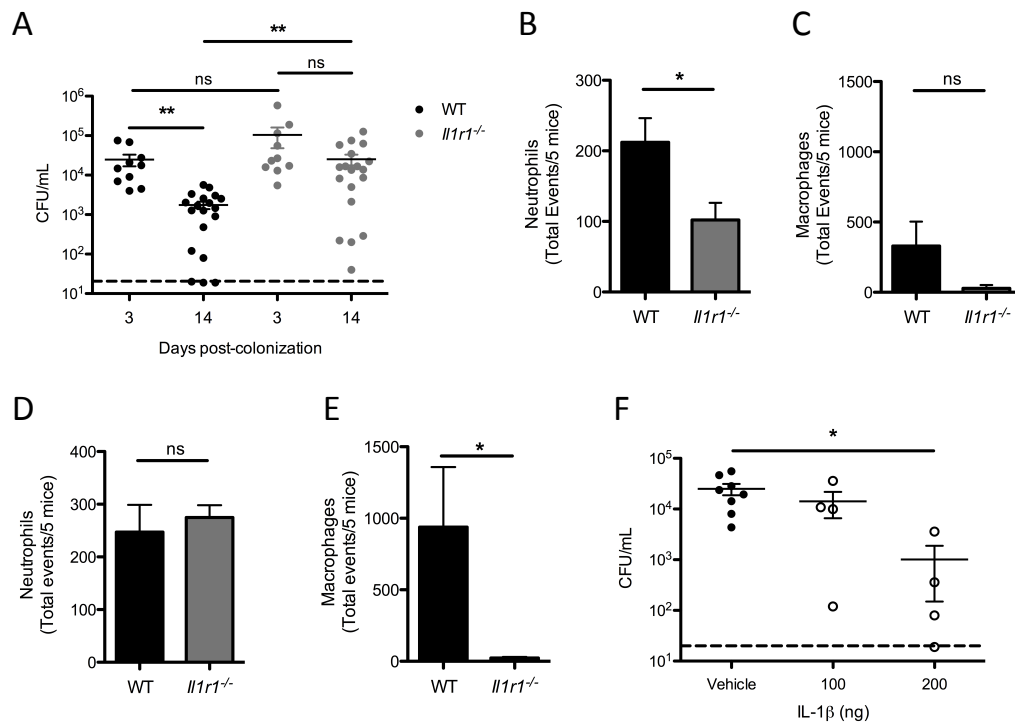
(A) Western blot of supernatants for the presence of caspase-1 (Casp-1) and interleukin-1 beta (IL-1 $\beta$ ). BMMs primed with 0.5  $\mu$ g/mL LPS and stimulated with ATP were used as a positive control (PC). Representative images shown.

(B) Amounts of IL-1 $\beta$  in supernatants were quantified by ELISA. Results are from 2 independent experiments, error bars represent  $\pm$  SEM, One-way ANOVA with Newman-Keuls post test, \*\*\*P < 0.001.



**Figure 3.2. Expression of IL-1 cytokines is up-regulated during *S. pneumoniae* colonization and is dependent on TLR2.**

(A-D) Wild type (black bars) or TLR2-deficient (*Tlr2*<sup>-/-</sup>, white bars) mice were intranasally inoculated with 10<sup>7</sup> CFU of *S. pneumoniae* (Spn). At 14 days post-colonization the upper respiratory tracts were lavaged with RLT RNA lysis buffer. Gene expression relative to PBS (Mock) inoculated mice was measured by quantitative RT-PCR for *Il1a* (A and C) and *Il1b* (B and D). Results are from at least 2 independent experiments, n > 10 mice, error bars represent ± SEM, Mann-Whitney U test, \*P < 0.05, \*\*\*P < 0.001, ns, not significant.



**Figure 3.3. Sensing of IL-1 family cytokines is important for bacterial clearance and macrophage recruitment.**

(A-E) Wild type (WT, black) or IL-1 receptor-deficient (*Il1r1*<sup>-/-</sup>, grey) mice were intranasally inoculated with 10<sup>7</sup> CFU of *S. pneumoniae* and at the time-points indicated PBS lavages of the upper respiratory tract were obtained.

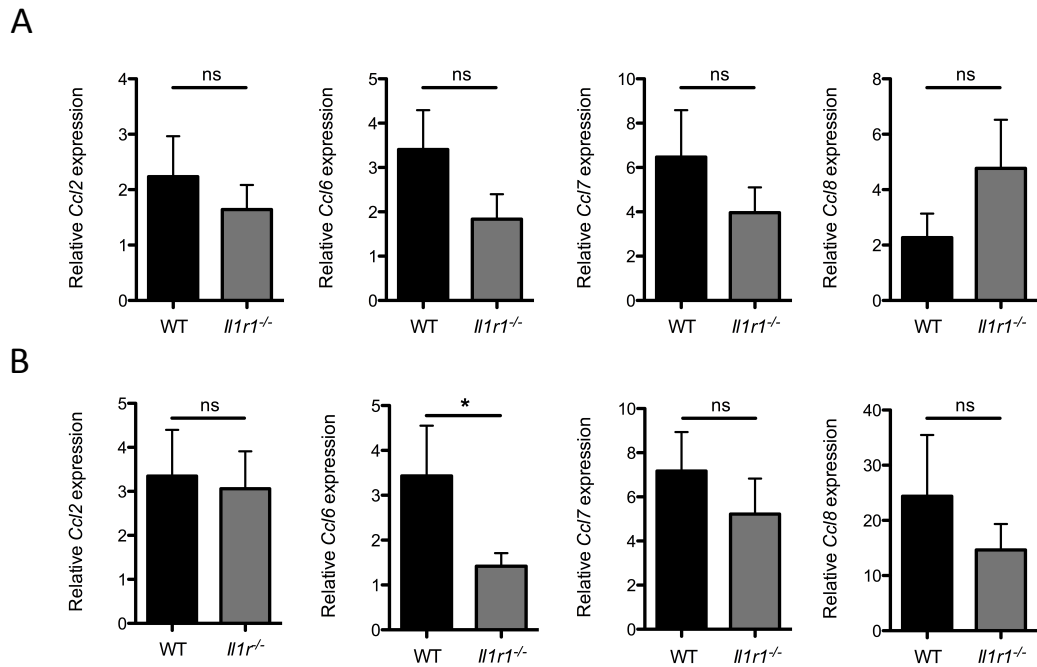
(A) Bacterial numbers of colonizing pneumococci were quantified by plating of nasal lavages. Dashed line indicates limit of detection. Results are from 2 to 4 independent experiments, n  $\geq$  10 mice per group, error bars represent  $\pm$  SEM, Kruskal-Wallis with Dunn's post-test, \*\*P < 0.01, ns, not significant.

(B-E) Cellular infiltrates in the nasal lavages of 5 mice were measured by flow cytometry. Quantification of neutrophils (Ly6G<sup>+</sup>, CD11b<sup>+</sup>) and monocytes/macrophages (F4/80<sup>+</sup>, CD11b<sup>+</sup>) after 3 (B and C) and 14 (D and E) days of colonization are shown. Results are from 4 independent experiments, error bars represent  $\pm$  SEM, Student's t-test or Mann-Whitney U test as appropriate, \*P < 0.05, ns, not significant.

**Figure 3.3 Continued.**

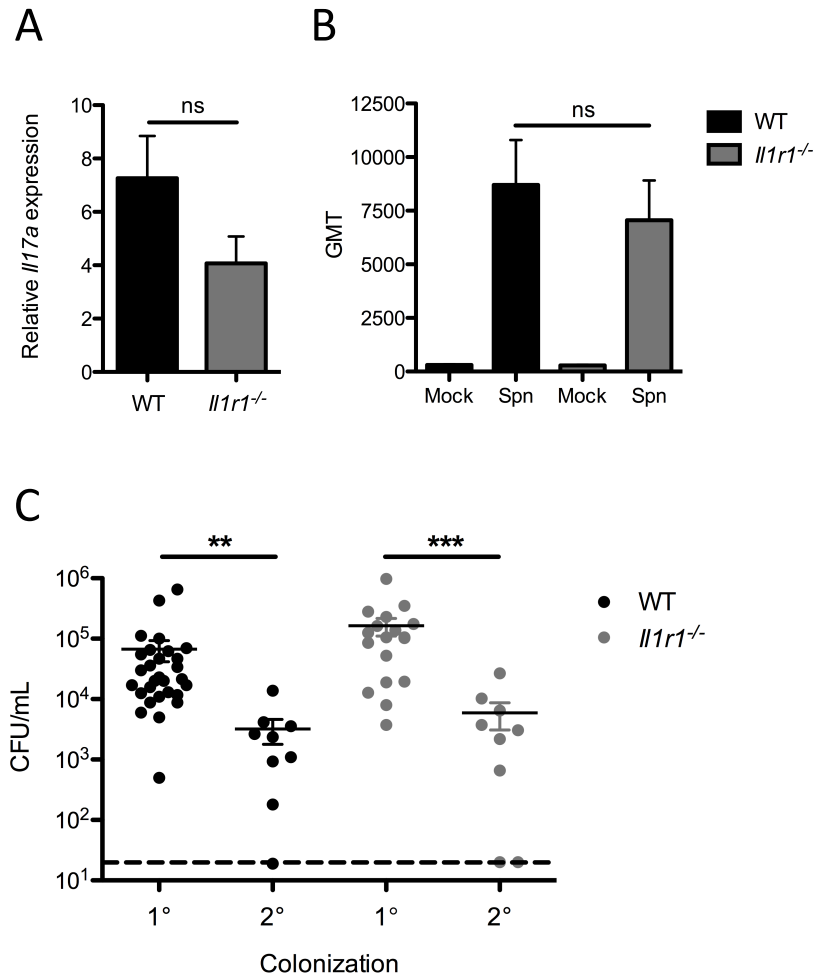
(F) WT mice were inoculated with  $10^7$  CFU of a pneumolysin-deficient *S. pneumoniae* strain and intranasally administered 100 or 200 ng of recombinant IL-1 $\beta$  (white circles) or vehicle control (PBS, black circles) every other day for 14 days. Nasal lavages were obtained and numbers of pneumococci were measured by plating serial dilutions. Dashed line indicates limit of detection. Results are from 2 experiments,  $n \geq 4$  mice per group, error bars represent  $\pm$  SEM, Kruskal-Wallis with Dunn's post-test, \* $P < 0.05$ .





**Figure 3.4. *Il1r1*<sup>-/-</sup> mice have altered C-C chemokine profiles during *S. pneumoniae* colonization.**

(A and B) Wild type (WT, black bars) or IL-1 receptor-deficient (*Il1r1*<sup>-/-</sup>, grey bars) mice were inoculated with 10<sup>7</sup> CFU of *S. pneumoniae*. At 3 (A) and 14 (B) days post-colonization RLT RNA lysis buffer lavages of the upper respiratory tract were obtained and gene expression of *Ccl2*, *Ccl6*, *Ccl7*, and *Ccl8* was measured by quantitative RT-PCR. Values are relative to PBS (Mock) inoculated mice. Results are from 2 to 4 independent experiments, n ≥ 9 mice, error bars represent ± SEM, Mann-Whitney U test, \*P < 0.05, \*\*P < 0.01, ns, not significant.



**Figure 3.5. Sensing of IL-1 does not alter adaptive immunity to the pneumococcus.**

(A and B) Wild type (WT, black bars) or IL-1 receptor-deficient (*Il1r1*<sup>-/-</sup>, grey bars) mice were intranasally colonized with *S. pneumoniae* (Spn) for 14 days.

(A) RLT RNA lysis buffer lavages of the respiratory tract were obtained and expression of *Il17a* was measured by quantitative RT-PCR. Values are reported as fold-change relative to PBS (Mock) inoculated mice. Student's t-test, ns, not significant.

(B) Total anti-pneumococcal serum IgG levels were measured by ELISA. Values are expressed as geometric mean titer (GMT), Kruskal-Wallis with Dunn's post-test, ns, not significant.

**Figure 3.5 Continued.**

(C) WT (black circles) or *Il1r1*<sup>-/-</sup> (grey circles) mice were inoculated with 10<sup>7</sup> CFU of *S. pneumoniae*. For primary (1°) colonization, mice were sacrificed at day 3 post-colonization, PBS nasal lavages obtained, and bacterial density measured by plating. For secondary (2°) colonization, mice were allowed 8 weeks to clear the pneumococci and then rechallenged with an isogenic *S. pneumoniae* strain distinguishable by antibiotic resistance. Nasal lavages were obtained at day 4 post-secondary colonization and bacterial density was quantified by plating. Dashed line indicates limit of detection. Results are from 2 to 5 independent experiments, n ≥ 10 mice, error bars represent ± SEM, Kruskal-Wallis with Dunn's post-test, \*\*P < 0.01, \*\*\*P < 0.001.

## **CHAPTER IV**

### **Materials and Methods**

## Bacterial Strains and Mutants

*S. pneumoniae* strains were grown overnight at 37°C on tryptic soy (TS) agar plates containing 5% sheep blood (BD) and the following day, liquid cultures were inoculated into broth TS and incubated in a nonshaking water bath at 37°C to mid-log phase (OD $\approx$ 0.5). The wild type 23F strain was originally isolated from an experimental human carriage study (173). Mutations in *pgdA* and *adr* (59) were introduced into previously described *S. pneumoniae* strains that were either deficient in or corrected for pneumolysin expression (60, 171) by transformation with chromosomal DNA, followed by selection for kanamycin (500  $\mu$ g/mL) and spectinomycin (200  $\mu$ g/mL) resistance, respectively. Sensitivity to lysozyme was phenotypically confirmed by *in vitro* bacterial lysis as previously described (59). The *ply<sub>TL-AA</sub>* mutation, containing previously characterized alanine mutations at the threonine and leucine residues responsible for binding cholesterol (73), was constructed using overlap extension PCR with primer pairs to introduce the two alanine mutations (Fwd 5'-TGG GGA ACA GCT GCC TAT CCT CAG GTA GAG GAT-3' and Rev 5'-ATC CTC TAC CTG AGG ATA GGC AGC TGT TCC CCA-3') and primers flanking the pneumolysin gene (Fwd 5'-AAA AAA GAA GCC GAT AAG GAA AAG ATG AGC G-3' and Rev 5'-GAA AGT TTC AGC CAA GTT TGA CAA AGT CAG CTC-3'). The amplified construct was introduced into a 23F pneumococcal strain containing a Janus Cassette (253) in place of the pneumolysin gene by transformation and homologous recombination. Transformants were selected for streptomycin resistance (200  $\mu$ g/mL) and

sensitivity to kanamycin (200  $\mu\text{g/mL}$ ) was confirmed by patching. Pneumococci were pelleted by centrifugation at 14,000 rpm and resuspended in phosphate buffered saline (PBS).

### **Hemolysis Assay**

As previously described (223), pellets of wild type (WT), pneumolysin-deficient (*ply*-), pneumolysin toxoid (*ply<sub>TL-AA</sub>*), or corrected (*ply*+) *S. pneumoniae* cultures were lysed in 400  $\mu\text{L}$  lysis buffer (0.01% sodium dodecyl sulfate, 0.1% sodium deoxycholate, and 0.015M sodium citrate) and incubated at 37°C for 30 min. Lysates were then transferred to a 96-well V-bottom plate and serially diluted three fold in DTT buffer (10 mM dithiothreitol, 0.1% bovine serum albumin in phosphate buffered saline). 2% horse red blood cells were added to each well and incubated at 37°C for 30 min. Plates were centrifuged for 10 min at 3000 rpm to pellet unlysed cells and imaged.

### **Cell Culture**

Bone-marrow cells harvested from the femurs and tibiae of C57BL/6, FVB/N, *LysM*<sup>-/-</sup>, and *Tlr4*<sup>-/-</sup> mice were differentiated into macrophages by culturing in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 30% L929 supernatant, and 20% FBS at 37°C with 5% CO<sub>2</sub> for 7-9 days. One day prior

to infection, macrophages were replated in DMEM supplemented with 15% L929 enriched supernatant and 10% FBS. All animal experiments were conducted according to the guidelines outlined by National Science Foundation Animal Welfare Requirements and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01, protocol number 803231.

### **Immunofluorescence Staining and Confocal Microscopy**

BMMs were seeded on to poly-L lysine-coated coverslips (BD) in a 24-well plate at a density of  $2.5 \times 10^5$  cells/slip and the following day, were incubated with non-opsonized *pgdA-/adr-* pneumococci at MOI=10 for 2.5 hours, washed with PBS, and fixed in 3% paraformaldehyde. Cells were quenched with 50 mM  $\text{NH}_4\text{Cl}$  and permeabilized with PGS (0.01% saponin, 0.25% gelatin, 0.02% sodium azide, in PBS). To detect pneumococcal capsular polysaccharide, the cells were incubated with type 23F rabbit serum (Statens Serum Institut) at a dilution of 1:5000 and detected with an anti-rabbit-Cy3 secondary antibody (Jackson ImmunoResearch) at a dilution of 1:600 in PGS. Phagosome membranes were labeled with a rat anti-Lamp-1 antibody (eBioscience) at a dilution of 1:500 in PGS and primary antibody was detected with an anti-rat Cy2-conjugated secondary antibody (Jackson

ImmunoResearch) at a dilution of 1:600 in PGS. Nuclei were stained with 0.5 ug/mL DAPI for 3 min. Slides were imaged on a Nikon Eclipse Ti-U spinning disk confocal microscope at the Molecular Pathology and Imaging Core at the University of Pennsylvania.

### **Electron Microscopy**

Macrophage samples were high pressure frozen in an Abra HPM-010 and freeze substituted in 99% acetone, 0.1% uranyl acetate, 1% dH<sub>2</sub>O in a Leica AFSII. Freeze substitution was performed at -90°C for 72 hours and then ramped to -50°C over 24 hours where cells were placed in four hour graded steps of 25%, 50%, 75%, 100% HM-20 in acetone. HM-20 was polymerized with 360 nm light for 48 hrs at -50°C and an additional 24 hours at room temperature. Polymerized blocks trimmed to regions of interest were cut at 60-80 nm thickness and immunologically probed with IgG purified type 23F rabbit serum. Protein A conjugated to 15 nm colloidal gold was used to secondarily detect the presence of antibody-antigen complexes. Imaging was performed on a JEOL 1010 TEM operating at 80 KeV.

### **Cellular Fractionation**

BMMs were seeded in a 6-well, non-tissue culture-treated plate at a density of  $1 \times 10^6$  cells/well. The following day, the cells were infected with non-



opsonized pneumococcal strains at MOI=50 and spun for 5 min at 3000 rpm. Where indicated, the BMMs were incubated with 20  $\mu$ M cytochalasin D (Sigma), 30 nM bafilomycin A, (Sigma) or 10  $\mu$ M SB203580 (Cell Signaling) at 37°C for 1 hour prior to infection. Following infection, the cells were incubated on ice for 1 hour, washed five times with PBS, and incubated for an additional 2 hours at 37°C. BMMs were washed three times with PBS, lifted with PBS containing 2 mM EDTA and permeabilized in 20  $\mu$ M digitonin (Sigma) to lyse the plasma membrane to generate cytosol fractions, or 0.1% saponin (Fluka) to lyse all membranes to generate whole cell fractions. Cells were spun at 15,000 g for 10 min and the resulting supernatants were ultracentrifuged at 4°C for 1 hour at 355,000 g. Supernatants were lyophilized and resuspended in dH<sub>2</sub>O.

### **Gentamicin Protection Assay**

BMMs were seeded in a 12-well plate at a density of  $4 \times 10^5$  cells/well and, the following day, infected with pneumococcal strains at MOI=50 and spun for 5 min at 3000 rpm. The BMMs were incubated on ice for 1 hour, then washed three times with PBS, and incubated at 37°C for 15 min for bacterial uptake. 300  $\mu$ g/ml gentamicin was then added to kill remaining extracellular bacteria. At the time points indicated, the cells were lysed with dH<sub>2</sub>O and serially diluted in PBS. Dilutions were plated on TS agar plates and incubated overnight at 37°C/5% CO<sub>2</sub>.

### **Cytotoxicity Assay**

BMMs were seeded in a 48-well plate at a density of  $2.5 \times 10^5$  cells/well and cultured overnight at 37°C. The following day the cells were primed with 400 ng/mL Pam3CSK4 for 4 hours and then infected with *S. pneumoniae* at MOI=10. At 2 hours post-infection the cell culture medium was replaced with DMEM containing 300 µg/mL gentamicin. Supernatants from infected macrophages were collected at 24 hours post-infection. Lactate dehydrogenase release was quantified using the Cytotoxicity Detection Kit Plus (Roche) per the instructions of the manufacturer.

### **ELISA for IL-1β**

Supernatants harvested at 24 hours post-infection were assayed for the presence of IL-1β using the ELISA MAX Mouse IL-1β Kit (BioLegend) per the instructions of the manufacturer.

### **Western Blotting**

Supernatants from BMMs infected with pneumococci were harvested at 24 hours post-infection, separated on a 10% Tris-SDS gel (BioRad) and detected by primary antibody incubation for Caspase-1 (Santa Cruz) at a dilution of 1:200 and IL-1 beta (AbCam) at a dilution of 1:2000. Sub-cellular fractions

from BMMs were validated by running resuspended supernatants on a 10% Tris-SDS gel (BioRad). Monoclonal anti-Lactate dehydrogenase and polyclonal anti-Cathepsin B antibodies (AbCam) were used for primary detection. Lysates from *S. pneumoniae* strains expressing pneumolysin mutations were separated on a 10% Tris-SDS gel and detected using a mouse monoclonal anti-pneumolysin primary antibody (Leica) and previously described (57) anti-pneumococcal surface protein A (PspA) mouse serum. Rabbit horseradish peroxidase (HRP) conjugated (GE Healthcare) and mouse HRP conjugated (GE Healthcare) antibodies were used for detection of the primary antibodies.

### **ELISA for Capsular Polysaccharide**

Sub-cellular fractions of infected BMMs were assayed for pneumococcal CPS by capture ELISA. Immulon 2HB plates (Thermo Scientific) were coated with type 23F rabbit serum at a dilution of 1:5000 and incubated overnight at room temperature (RT). The plate was incubated with serial dilutions of samples or a type 23F CPS standard (ATCC) for 2 hours at RT. Samples and standards were detected with a monoclonal anti-23F CPS antibody at a 1:300 dilution for 2 hours at RT. Monoclonal antibody was detected with a goat anti-mouse alkaline phosphatase antibody (Sigma) at a 1:10000 dilution for 2 hours at RT. The plate was developed with phosphatase substrate (Sigma) for 30 min and read at OD=415nm.

### **Murine Model of *S. pneumoniae* Nasopharyngeal Colonization**

C57BL/6 (WT) and *Tlr2*<sup>-/-</sup> mice were obtained from The Jackson Laboratory. *Il1r1*<sup>-/-</sup> mice (89) were a generous gift from Sunny Shin (University of Pennsylvania). 10<sup>7</sup> colony forming units (CFU) of *S. pneumoniae* in 10 µL PBS were inoculated into the nares of unanesthetized mice. Inocula were serially diluted in PBS and grown overnight on TS agar plates to verify dose. At the time points indicated in the figures, the mice were sacrificed, the trachea cannulated, and lavaged with 200 µL PBS through the nares. The resulting lavage fluid was plated in serial dilutions on TS agar plates, grown overnight at 37°C/5% CO<sub>2</sub>, and the following day CFU counted. For intranasal administration of cytokine, WT mice colonized with a pneumolysin-deficient *S. pneumoniae* strain (222) received 100 or 200 ng of recombinant IL-1β (eBioscience) resuspended in 10 µL PBS or 10 µL PBS alone as a vehicle control every other day for 14 days. For secondary challenge experiments, mice of the indicated genotype were colonized with WT *S. pneumoniae* and allowed 8 weeks to clear colonization. They were then re-colonized with an isogenic strain containing a single point mutation that confers resistance to streptomycin and has no colonization defect (63, 253).

### **Quantitative RT-PCR**

Following lavage with PBS, sacrificed mice were lavaged with 600 µL RLT

RNA Lysis buffer (QIAGEN) containing 10% 2-mercaptoethanol. Samples were stored at -80°C until RNA was isolated using a QIAshredder Kit (QIAGEN) followed by an RNeasy Mini Kit (QIAGEN) per the manufacturers protocol. Complementary DNA (cDNA) was reverse transcribed using a high-capacity cDNA kit (Applied Biosystems). 10 ng cDNA was used as a template in each reaction with 0.5 µM forward and reverse primers and Power SYBR Green (Applied Biosystems) per the manufacturers protocol. Reactions were amplified with the StepOnePlus Real-Time PCR system (Applied Biosystems) and comparisons were calculated using the  $\Delta\Delta C_T$  method. The following primers were used for amplification: GAPDH-F: 5'-AGG TCG GTG TGA ACG GAT TTG-3'; GAPDH-R: 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'; IL-1A-F: 5'-GCA CCT TAC ACC TAC CAG AGT-3'; IL-1A-R: 5'-TGC AGG TCA TTT AAC CAA GTG G-3'; IL-1B-F: 5'-GCA ACT GTT CCT GAA CTC AAC T-3'; IL-1B-R: 5'-ATC TTT TGG GGT CCG TCA ACT-3'; CCL2-F: 5'-AGC TCT CTC TTC CTC CAC CAC-3'; CCL2-R: 5'-CGT TAA CTG CAT CTG GCT GA-3'; CCL6-F: 5'-ATG AGA AAC TCC AAG ACT GCC-3'; CCL6-R: 5'-TTA TTG GAG GGT TAT AGC GAC G-3'; CCL7-F: 5'-GCT GCT TTC AGC ATC CAA GTG-3'; CCL7-R: 5'-CCA GGG ACA CCG ACT ACT G-3'; CCL8-F: 5'-TCT ACG CAG TGC TTC TTT GCC-3'; CCL8-R: 5'-AAG GGG GAT CTT CAG CTT TAG TA-3'; IL-17A-F: 5'-TTT AAC TCC CTT GGC GCA AAA-3'; IL-17A-R: 5'-CTT TCC CTC CGC ATT GAC AC-3'

### **Flow Cytometry**

PBS lavages from 5 mice were pooled per group, pelleted at 1500 rpm for 10 min and resuspended in PBS containing 1% bovine serum albumin (BSA). Samples were first blocked for 10 min in 1% BSA (Sigma-Aldrich) and then blocked again with a rat anti-mouse FcγIII/II receptor antibody (BD). Cells were stained for 30 min at 4°C with a cocktail of the following rat anti-mouse antibodies: CD4-FITC (BD), Ly6G-PE (BioLegend), Cd11b-PerCP cy5.5 (BioLegend), and F4/80-APC (eBioscience).

### **Quantification of Anti-pneumococcal Serum IgG**

The wild type 23F strain used for colonization was grown to mid-log phase in TS, pelleted, and resuspended in coating buffer (0.015 M Na<sub>2</sub>CO<sub>3</sub>, 0.035 M NaHCO<sub>3</sub>, pH 9.6) to OD<sub>620</sub>=0.1. Immulon 2HB 96-well plates (Thermo) were coated with pneumococci by incubating 100 μL per well of resuspension overnight at 4°C. Plates were blocked for 1 hr at 37°C in 1% BSA in PBS and washed with PBS containing 0.05% Brij-35 (Fisher). Serum samples were serially diluted 2-fold and incubated overnight at 4°C. The plates were washed with 0.05% Brij-35 in PBS and pneumococcal-specific antibodies were detected by incubation with a goat anti-mouse IgG alkaline phosphatase conjugated antibody for 1.5 hrs at room temperature. Plates were developed for 1 hr at 37°C using phosphatase substrate (Sigma) and read at 415 nm. Geometric mean titers were calculated based on the sample

dilution where absorbance = 0.1.

## **CHAPTER V**

### **Summary and Discussion**



### **Cytosolic access is dependent on phagocytosis and lysozyme**

Our studies define several host factors that are required for *S. pneumoniae* killing and subsequent cytosolic access of pneumococcal components. Previous work from our lab indicates that this phenomenon occurs *in vivo* (60) however, our studies investigated cytosolic access in an *in vitro* cell culture system. This was necessary because macrophages in the upper respiratory tract are of low abundance and remain poorly characterized. Working in a cell culture system however, allowed us to use high enough cell numbers that we could quantify cytosolic contents, as well as employ pharmacologic agents and genetic knock-outs to better dissect the mechanism of cytosolic access of pneumococcal components. Phagocytosis is the first step in this host-pathogen interaction and inhibition of bacterial uptake prevents pneumococcal components from accessing the cytosol. The scavenger receptor MARCO promotes both bacterial uptake and Nod2-dependent sensing of pneumococci *in vitro* and MARCO-deficient mice have impaired clearance of *S. pneumoniae* colonization (67). This indicates that phagocytosis of pneumococci by macrophages in non-immune hosts occurs through bacterial binding of this scavenger receptor, which leads to cytosolic access that triggers Nod2-dependent innate immune signaling cascades.

Once inside the phagosome, pneumococci are subject to a battery of host defenses, including acidification, proteases, and reactive oxygen species from the respiratory burst. Previous studies have determined that nitric oxide contributes to pneumococcal killing by macrophages (167) and our findings

further characterize how the host cell achieves destruction of the pneumococcus. Our data show that host expression of the muramidase lysozyme is required for killing of *S. pneumoniae* (Fig. 2.2E) and that while cell wall modifications, which confer bacterial resistance to lysozyme on the mucosal surface (59), can delay pneumococcal killing by macrophages (Fig. 2.2D), interaction with these professional phagocytes is still fatal for the pneumococcus. We further observe that access to the cytosol is independent of phagosome acidification (Fig. 2.5D). This finding is consistent with lysozyme-mediated degradation and killing, as this muramidase is enzymatically active at neutral pH (60).

TLR4 has been reported to directly sense the pneumococcal pore-forming toxin pneumolysin *in vitro*, although the requirement for TLR4 signaling in protection from pneumococcal colonization and invasive disease remains a subject of debate (19, 65, 162). Our observation that TLR4 is not required for cytosolic access (Fig. 2.5B) is consistent with other studies investigating intracellular signaling pathways that are activated by pneumococcal infection. Pneumolysin triggers NLRP3-dependent inflammasome activation independently of TLR4 (175), and cytosolic access of pneumococcal DNA activates type I interferon signaling without contribution from TLR4 (208). Given that TLR4 signaling may defend against *S. pneumoniae* colonization (162), it remains possible that TLR4 is required for host defense against pneumolysin activity in epithelial cells rather than in professional phagocytes.

### **Cytosolic access requires pneumolysin**

In these studies, we define a critical role for the pneumococcal pore-forming toxin pneumolysin in mediating transit of bacterial components to the cytosol of macrophages. We show that a pneumolysin-deficient pneumococcal strain has significantly impaired cytosolic access (Fig. 2.4B) and furthermore, the ability of pneumococcal components to access the cytosol requires pneumolysin binding of cholesterol in host membranes (Fig. 2.4B). We were unable to ascertain whether pore-formation is specifically required for cytosolic access because a pneumococcal strain expressing a pneumolysin pore-forming mutant had an intermediate phenotype in our fractionation assay (data not shown). Pore-formation by pneumolysin is usually characterized by a hemolysis assay that measures the ability of the toxin to lyse horse red blood cells. While the pore-formation mutant is almost completely attenuated in this assay (144, 223, 239), we are unable to say whether there is still residual toxin activity in the context of pneumococcal degradation by professional phagocytes.

Our finding that cytosolic access requires both expression of the pneumolysin toxin and its ability to bind host membranes provides further insight into how a host discriminates between commensal and potentially pathogenic microbes. Pneumolysin can facilitate transit of peptidoglycan to the cytosol of epithelial cells during co-infection with other bacterial species (222) and other members of the CDC toxin family are well known to

translocate effector proteins (160) or provide cytosolic access to live microbes (17, 216) as part of an organisms' survival. In contrast, the action of pneumolysin on the host cell coincides with bacterial lysis and does not directly benefit the bacterium that releases the toxin. Recent work has characterized intracellular innate immune detection of other extracellular pathogens that is dependent on expression of their CDC toxins (97), suggesting that toxin-mediated escape of degraded bacterial fragments from the phagosome likely occurs for other microbes.

Additional mechanisms of cytosolic access of bacterial components have been described. A pneumococcal strain expressing a pneumolysin pore-forming mutant causes lysosome destabilization at late time points (24 hours post-infection) (25), though exactly how this occurs is unresolved. Studies investigating the transit of MDP, the minimal Nod2 ligand (88), to the host cell cytosol have shown a critical role for the peptide transporters SLC15A3 and SLC15A4 (196). Unlike previous reports linking the peptide transporters PEPT1 and PEPT2 to the transit of MDP to the cytosol (254, 269), this study further demonstrates the presence of these transporters and Nod2 on the phagosome membrane during bacterial infection (196). The possible contributions of SLC15A3 and SLC15A4 to cytosolic access of pathogens that express pore-forming toxins or do not survive in the phagosome provides an opportunity for future investigation, which could reveal that more than one route to the cytosol exists for the bacterial ligands that activate innate immune sensors.

## **Cytosolic access results in pro-inflammatory cell death and drives clearance of colonization**

Our observation that inhibition of p38 MAPK increases cytosolic access of pneumococcal components (Fig. 2.6B) shows that the host cell is able to sense and defend against the action of pneumolysin. P38 MAPK phosphorylation occurs rapidly in macrophages following incubation with pneumococci (58), suggesting that this sensing occurs at the plasma membrane, while the sequelae of p38 MAPK activation limits transit of bacterial products across the phagosome membrane. Although the host cell mounts this defense, accessing the cytosolic compartment results in death of the host cell. Our data demonstrate that this cytotoxicity is dependent on both bacterial uptake and expression of pneumolysin (Fig. 2.7). This 'mutually assured destruction' – death of both the pneumococcus and the macrophage – may be a strategy by the bacterium to counteract the host cells responsible for its clearance.

In these studies, we also show that host cell death results in IL-1 $\beta$  release *in vitro* and similar to cell death, is dependent on phagocytosis and bacterial expression of pneumolysin (Fig. 3.1B). This observation complements results in the literature reporting inflammasome activation and IL-1 $\beta$  release with a different pneumococcal strain in a variety of cell types (72, 133, 175). While we were unable to measure levels of mature cytokine *in vivo*, *Il1r1*<sup>-/-</sup> mice have delayed clearance of colonization (Fig. 3.3A), which correlates with lower numbers of macrophages in the nasopharynx (Fig.

3.3E), suggesting that sensing of IL-1 cytokines that results from cytosolic access contribute to bacterial clearance. However, our results demonstrate that expression of *I/1a* and *I/1b* increases upon colonization in a manner that is dependent on TLR signaling (Fig. 3.2), a finding that is consistent with cellular priming for inflammasome activation.

Previous work from our lab defined a critical role for the intracellular host receptor Nod2 in clearance of pneumococcal colonization (60). Interestingly, these studies found that clearance of colonization was most significantly impaired in mice deficient in both Nod2 and TLR2, suggesting redundancy between these two pattern recognition receptors. Our studies define an additional intracellular innate immune pathway that is dependent on TLR stimulation, which is activated by *S. pneumoniae* and contributes to bacterial clearance from the host. It is therefore possible that both Nod2 sensing and IL-1 cytokines contribute to clearance of pneumococci from the nasopharynx and that cytosolic access is the unifying event that triggers both of these host sensing mechanisms.

Activating multiple innate immune pathways may also have significant contributions to host protection from pneumococcal disease. Children with deficiencies in either MYD88 or IRAK-4, molecules that are downstream of TLRs and the IL-1 receptor, experience recurrent pneumococcal disease (21, 146, 213) that cannot be attributed to loss of TLR function alone (182). This implies that sensing of IL-1 cytokines, in addition to TLR activation, may have a critical role in defense against *S. pneumoniae*.

Why the pneumococcus would make a toxin that activates innate immune signaling that drives the host to clear it from its niche is a logical question resulting from these studies. Our observation that IL-1 sensing promotes inflammation in the nasopharynx without further contributing to the development of adaptive immune responses may provide some answers. The host inflammatory response to pneumococcal colonization promotes both bacterial growth (247) and transmission (244); therefore, production of IL-1 cytokines during colonization may contribute to inflammation that promotes the pneumococcus' lifestyle without impacting its ability to re-colonize the same host at a later time.

### **Model of cytosolic access of pneumococcal components**

Based on our studies interrogating the mechanism by which pneumococcal components access the host cell cytosol, we propose a model of cytosolic access with critical roles for bacterial degradation by lysozyme and the function of the pore-forming toxin pneumolysin (Fig. 4.1). The presence of bacterial products in the cytosol is dependent on phagocytosis, indicating that bacterial uptake initiates this host-pathogen interaction. Killing of *S. pneumoniae* occurs rapidly following phagocytosis and pneumococcal degradation is delayed by bacterial modifications that confer resistance to lysozyme or in macrophages that are lysozyme-deficient. These findings demonstrate that lysozyme significantly contributes to bacterial

killing by macrophages. A lysozyme sensitive pneumococcal strain has increased amounts of bacterial components in the host cell cytosol, indicating that lysozyme degradation is also important for generating bacterial fragments that transit to the cytosol. Lysis of the bacterial cell causes the release of the pore-forming toxin pneumolysin, which is required for pneumococcal components to access the cytosol. Additionally, the ability of the toxin to bind cholesterol in the host membrane is necessary for cytosolic access to occur, demonstrating a critical role for toxin function in this phenomenon. The host is able to detect and limit toxin activity through p38 MAPK activation, though ultimately these defenses are insufficient and the macrophage undergoes a pro-inflammatory cell death. Death of the macrophage results in the release of IL-1 $\beta$  and sensing of IL-1 family cytokines is required for successful macrophage recruitment and clearance of colonization (Fig. 4.2). The mechanism by which IL-1 cytokines promote macrophage-driven bacterial clearance is unknown, but provides an avenue of future inquiry.

## **Future Directions**

### *The role of p38 MAPK activation in vivo*

Our finding that p38 MAPK sensing of pneumolysin limits cytosolic access further contributes to the body of evidence that this signaling pathway is critical for defense against pore-forming toxins (116, 118, 223). Exactly



how p38 MAPK activation achieves this defense of the host cell is unclear and could be the subject of future investigation. Furthermore, the significance of p38 MAPK activation during pneumococcal colonization *in vivo* remains to be addressed. Studies of pneumococcal pneumonia have shown that p38 MAPK activation is critical for controlling bacterial burden (266), suggesting an important role for this signaling cascade in bacterial clearance at the mucosal surface and also demonstrating that inhibition of p38 MAPK can be achieved *in vivo*. However, the contribution of pneumolysin to p38 MAPK activation was not addressed in this study. In addition to pore-forming toxins, TLRs can activate P38 MAPK signaling and the contribution of pneumolysin or other pathogen associated molecular patterns in p38 MAPK-mediated defense during pneumococcal disease or colonization is still an open question.

*Determine the contributions of IL-1 $\alpha$  and IL-1 $\beta$  to clearance of colonization*

Our findings define a critical role for sensing by the IL-1 receptor in detection and clearance of pneumococcal colonization, although we have not demonstrated which IL-1 receptor agonists are responsible for this phenotype. The type 1 IL-1 receptor is activated by binding of both IL-1 $\alpha$  and IL-1 $\beta$  (84). While we show that IL-1 $\beta$  expression is highly induced during colonization (Fig. 3.2B) and administration of recombinant IL-1 $\beta$  is sufficient to promote a reduction in bacterial carriage (Fig. 3.3F), we have not excluded a potential contribution of IL-1 $\alpha$  in the host immune response to pneumococcal colonization. Attempts to neutralize IL-1 $\alpha$  and IL- $\beta$  in

colonized mice by intraperitoneal injection of monoclonal antibodies at concentrations that are effective on the mucosal surface (42) did not significantly alter clearance of colonization at 14 days post-infection (data not shown). Nasal lavages are highly diluted and we could not detect cytokines in samples from the upper respiratory tract; therefore, we were unable to confirm cytokine depletion upon treatment with the neutralizing antibodies.

To specifically address which IL-1 receptor ligands contribute to bacterial clearance from the nasopharynx, we could examine the dynamics of colonization in IL-1 $\alpha$  or IL-1 $\beta$ -deficient mice (112, 113, 128). Following inoculation of these mice with *S. pneumoniae*, we would be able to assess bacterial burden and immune cell infiltrate over time and determine whether the absence of IL-1 $\alpha$  or IL-1 $\beta$  alone phenocopies our findings from *Il1r1*<sup>-/-</sup> mice. We could also generate mice that lack both IL-1 $\alpha$  and IL-1 $\beta$  to determine if these cytokines have redundant or synergistic contributions to clearance of colonization. These experiments would clarify the individual contributions of both IL-1 $\alpha$  and IL-1 $\beta$  to the host immune response to *S. pneumoniae* colonization.

#### *Characterize the role of CCL6 in S. pneumoniae colonization*

Our observation that *Il1r1*<sup>-/-</sup> mice have lower expression of the macrophage chemokine CCL6 than WT mice during colonization gives rise to several avenues of further inquiry. Neutralization of CCL6 with depleting antibodies in colonized WT mice could show whether CCL6 is necessary for

clearance of pneumococci. This experiment, however, may have similar technical challenges in verifying depletion as the IL-1 $\alpha$  and IL-1 $\beta$  depletion experiments we have previously performed. Although CCL6 is a known macrophage attractant (147), it will be important to demonstrate if CCL6 is sufficient to recruit macrophages in the environment of the upper respiratory tract. To address this, we could intranasally administer exogenous CCL6 to colonized *Il1r1*<sup>-/-</sup> mice and monitor the presence of immune cell infiltrates, specifically the population of monocytes/macrophages, in lavages of the nasopharynx. We could also monitor whether administration of recombinant cytokine has an effect on bacterial clearance.

Determining which cells in the respiratory tract are the source of CCL6 is another question that remains to be addressed. CCL6 is produced by cultured macrophages (147, 207), as well as epithelial cells of the gut (145), making both of these cell types potential candidates. Furthermore, mRNA present in RLT lysis lavages of the upper respiratory tract is predominantly derived from epithelial cells of the mucosal barrier, suggesting that they are likely to be a source of CCL6. To test this hypothesis, we could section the upper respiratory tracts of mock infected or colonized mice and conduct immunofluorescence staining and imaging for the presence of CCL6. As CCL6 is a secreted protein, it may still be difficult to detect this chemokine within any cell population *in vivo*, in which case, *in vitro* cell culture could be informative.

To address the potential contribution of macrophages to the production

of CCL6, we could infect bone marrow-derived macrophages with *S. pneumoniae* and monitor for the transcription and production of C-C motif chemokines, including CCL6. Similar experiments have characterized CCL2 production by macrophages (60), and macrophages elicited in a model of irritant peritonitis are sources of CCL6 at later time points (147, 285).

CCL6 has been identified only in rodents and has two human homologs, CCL14 and CCL15, which share 36 and 53 percent amino acid identity with CCL6, respectively (145). The lack of a clear homolog in humans is a limitation of using a murine model. Therefore, determining whether either, or both, CCL14 and CCL15 are expressed as a result of *S. pneumoniae* sensing will be important for relating our findings to human carriage or disease. If prior experiments in murine cells show that macrophages produce and secrete CCL6 upon pneumococcal infection, we could perform similar experiments with human macrophages differentiated from blood monocytes and assay for both CCL14 and CCL15. Alternatively, we could use the monocyte derived THP1 cell line, that can phagocytose and kill pneumococci (225). Studies investigating chemokine production during *S. pneumoniae* infection have characterized a role for macrophage-produced IL-1 $\beta$  in driving production of the neutrophil chemokine CXCL8 by epithelial cells (168). We could conduct co-culture experiments *in vitro* to address the possibility that macrophage infection with *S. pneumoniae* results in production of IL-1 cytokines that are sensed by the epithelium, which then drives CCL14 or CCL15 expression and secretion. Together, these

experiments will better define the relationship between *S. pneumoniae* colonization, IL-1 family cytokines, and chemokine production.

*Determine the contribution of cytosolic access in colonization of other mucosal pathogens*

Ascertaining whether our findings can be generalized to other extracellular pathogens is a broader area of possible future inquiry. The implications for carriage of *Staphylococcus aureus* are of specific interest, particularly because *S. aureus* is second only to *S. pneumoniae* as a cause of pyogenic infections in MYD88 and IRAK-4-deficient patients (146). Similar to the pneumococcus, *S. aureus* is a colonizer of the host mucosal surfaces and can exist as both a pathogen and a commensal. While *S. aureus* can be isolated from non-mucosal surfaces such as the skin and frequently causes wound and soft tissue infections, the anterior nasopharynx is the most common site of colonization in humans (279). Carriage in the nasopharynx is believed to be the reservoir for colonization of other body sites and the source of disease (69).

There is evidence that cytosolic access of *S. aureus* bacterial components occurs during infection and is important for host defense. Nod2 sensing is dependent on *S. aureus* expression of the pore-forming  $\alpha$ -toxin and is critical for host control of cutaneous infection (115). Furthermore, *S. aureus* activates the NLRP3 inflammasome in macrophages in a manner that is dependent on lysozyme (243), though this pro-inflammatory cell death

may contribute to immunopathogenesis during pulmonary infection (135). The role of this innate immune recognition to *S. aureus* colonization and carriage is unknown.

Colonization by *S. aureus* has historically been challenging to study due to the paucity of identified strains that can establish and persist in mouse models (154). Recently, studies have identified strains that colonize the murine anterior nasopharynx for a duration of weeks (5, 111), allowing an opportunity to better examine the role of host innate immune responses to *S. aureus* carriage.

## **Concluding Remarks**

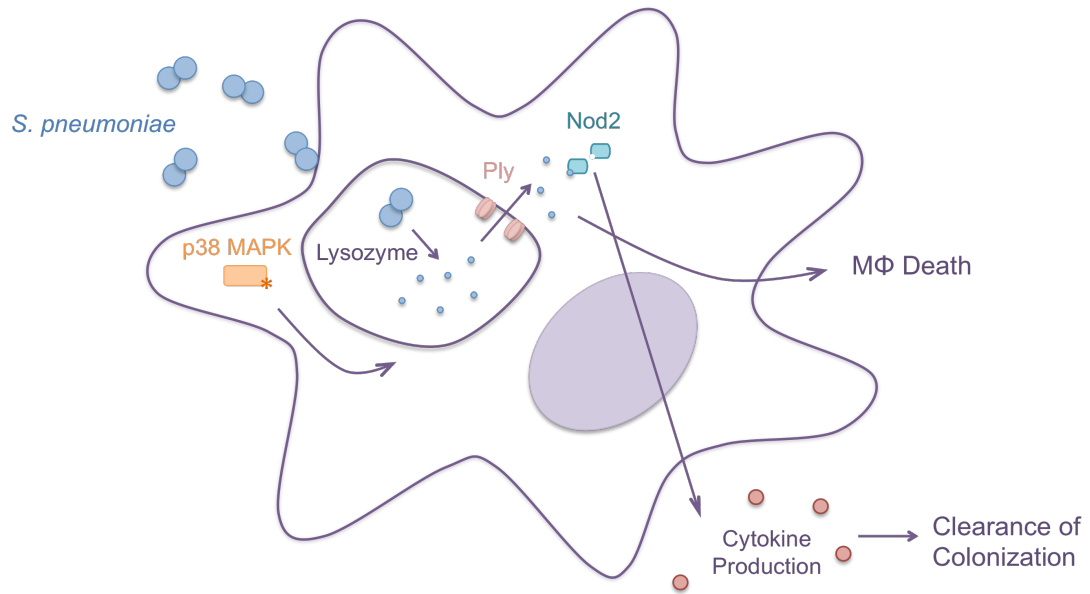
Accessing the cytosol is considered a conserved motif among pathogenic organisms (268) and host detection of cytosolic access is essential for discrimination between a pathogen and a commensal. Intriguingly, *Streptococcus pneumoniae*, a leading extracellular pathogen, activates intracellular innate immune signaling pathways, which contributes to host defense against colonization and disease by this organism. The studies presented here focused on the mechanism and consequences of cytosolic access by *S. pneumoniae*.

While many microbes access the cytosol to promote their replication, cytosolic access of pneumococcal components follows a fatal event for the pneumococcus. Our studies show that bacterial products access the cytosol in

a manner that is dependent on the pore-forming toxin pneumolysin and suggest that lysozyme degradation in the phagosome triggers toxin release. Detection of the virulence activity of a pore-forming toxin, even if it is produced by a microbe a phagocyte has killed, may provide the host a signal that this organism is potentially pathogenic and should be cleared although it is not causing disease.

We further demonstrate that cytosolic access results in phagocyte death and release of cytokines that promote inflammation, immune cell recruitment, and bacterial clearance, but do not contribute to the development of adaptive immunity. Inflammatory responses in the nasopharynx may promote pneumococcal growth and transmission and our findings provide important new insights into the dynamics of the pneumococcus' lifestyle and the potential contribution of its pore-forming toxin pneumolysin.

A

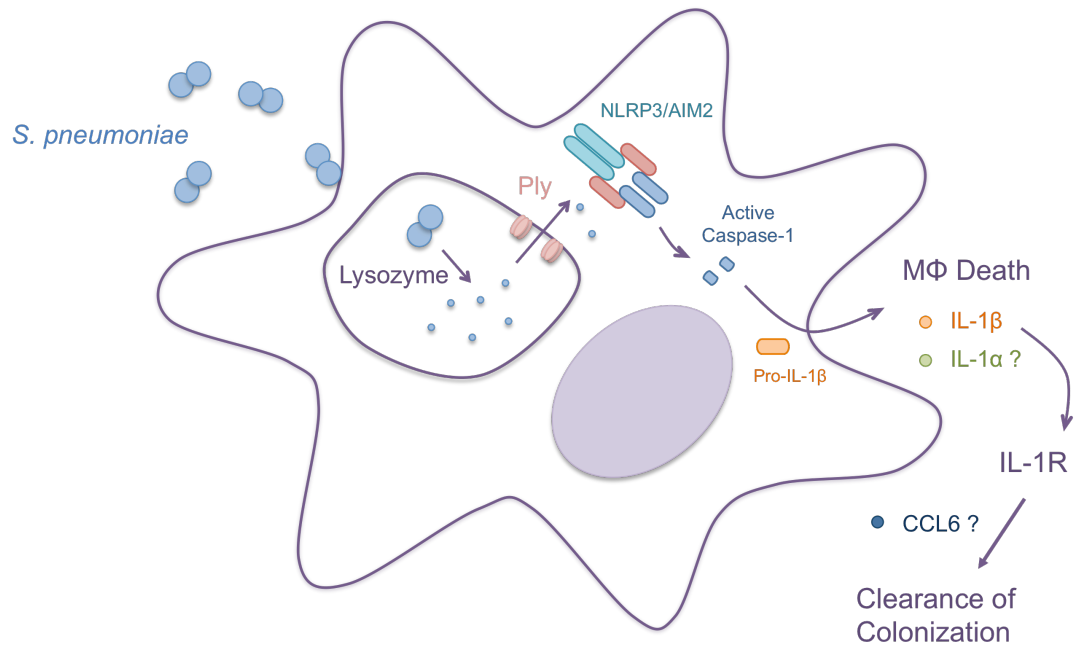


**Figure 4.1. Model of cytosolic access of pneumococcal components.**

(A) *S. pneumoniae* is phagocytosed and subsequently degraded by lysozyme. This releases the pore-forming toxin pneumolysin (Ply), which facilitates transit of bacterial components across the phagosome membrane to the cytosol. There, they activate Nod2 signaling, resulting in cytokine production and eventually clearance of colonization. Host sensing of Ply activates p38 MAPK signaling, which limits cytosolic access however, this phenomenon is fatal for the macrophage (MΦ) and results in host cell death.



A



**Figure 4.2. Model of IL-1 sensing and clearance of pneumococcal colonization.**

(A) Cytosolic access of pneumococcal components occurs after degradation by lysozyme and release of pneumolysin (Ply). This event triggers inflammasome (NLRP3 and AIM2) formation that leads to activation of caspase-1 and secretion of interleukin-1 beta (IL-β) and possibly interleukin-1 alpha (IL-1α). Sensing of IL-1 cytokines by the IL-1 receptor (IL-1R) promotes clearance of *S. pneumoniae* colonization, potentially through increased levels of the macrophage attractant C-C motif chemokine 6 (CCL6).

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